White Adipose as a Target for the Gastrointestinal Hormones

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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ABBREVIATIONS

AA	Arachidonic acid
AGRP	Ggouti related peptide
α-LA	α-linolenic acid
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
ASP	Acylation-stimulation protein
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
C/EBPα	CCAAT/enhancer binding protein-a
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
cDNA	Complementary DNA
CDS	Protein coding sequence
CLA	Conjugatea linoleic acid
CNS	Central nervous system
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CT	Computed tomography
CVA	Cardiovascular disease
DHA	Docosahexaenoic acid
DMED	Dulbecco's Modified Eagle Medium
DMN	Dorsomedial nucleus
Dnase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FCS	Foetal calf serum

FFA	Free fatty acid
FIAF	Fasting-induced adipose factor
FIZZ	Found in inflammatory zone
GHS-R	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GIP R	Glucose-dependent insulinotropic peptide Receptor
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1Receptor
GLUT	Glucose transporter
GPR-39	G-protein coupled receptor 39
HDL	High-density lipoprotein
HEPES	N-2-hydroxythylpiperazine-N' -2-ethanesulphonic acid
HIF-1α	Hypoxia-induced factor-l α
HIV	Human irmmunodeficiency virus
hMADS	Human multipotent adipose-derived stem cells
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-I-methylxanthine
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
JAK	Janus kinase
LA	Linoleic acid
LCFA	Long-chain fatty acid
LHA	Lateral hypothalamic area
LMF	Lipid mobilising factor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCR	Melanocortin receptor
MCP-1	Monocyte chemoattractant protein-l

MHC	Major Histocompatibility Complex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSH	Melanocyte stimulating hormone
NASH	Non-alcoholic steatohepatitis
NAFLD	Non-alcoholic fatty acid liver disease
NEFA	Non-esterified fatty acid
NFκB	Nuclear factor kappa-B
NGF	Nerve growth factor
NPY	Neuropeptide Y
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-l
PBEF	Pre-B cell colony-enhancing factor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PG	Prostaglandin
PIF	Proteolysis-inducing factor
PKB	Protein kinase B
PLA2	Phospholipase A
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acid
PVN	Paraventricular Nucleus
PYY	Peptide YY
RBP	Retinol biding protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
SAA	Serum amyloid A
SDS	Sodium dodecyl sulphate

S.E.M	Standard error of the mean
SGBS	Simpson-Golabi-Behmel syndrome
SNS	Sympathetic nervous system
SOCS3	Suppressor of cytokine signaling-3
SREBP-1c	Sterol regulatory element binding protein-1c
STAT	Signal transducer and activator of transcription
SV	Stromal vascular
T3	Triiodothyronine
Ta	Annealing temperature
TAG	Triaclyglycerol
TBE	Tris-borate-EDTA
TBS	Tris buffered saline
TEMED	N,N,N,N - tetramethylethy nediamine
TLRs	Toll-like receptors
Tm	Melting temperature
TNF-α	Tumour necrosis factor-α
Tris-HCl	Tris (hydroxymethyl) aminomethane Hydrochloride
TZD	Thiazolidinedione
UCP	Uncoupling protein
VEGF	Endothelial growth factor
VLDL	Very low-density lipoproteins
VMN	Ventromedial nucleus
WAT	White adipose tissue
ZAG	Zinc-a2-glycoprotein

DECLARATION

I declare that the content of this thesis entitled 'White Adipose as a Target for the Gastrointestinal Hormones' is my own work carried out at both the obesity Biology unit and the clinical biochemistry department at the University of Liverpool.

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July 2008

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ABSTRACT

Excessive fat deposition in the form of obesity is now a major health problem worldwide leading to an increased risk of a number of diseases, including type II diabetes, coronary heart disease and cancer. Adipose tissue was originally thought to be used by the body solely as an energy store. In recent years, increases in the prevalence of obesity and ongoing research, has shown that lipid storage is not the only function of white fat. The discovery of leptin, which was followed by the identification of other protein factors, termed 'adipokines', secreted by white adipose tissue (WAT), indicated that WAT is major endocrine organ.

The gastrointestinal tract is the portal for the entry of dietary nutrients and a number of intestinal hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), known as incretin's, and ghrelin, are involved in their absorption and storage. Recently, it was found that the gene precursor of ghrelin encoded another secreted and bioactive peptide named obestatin, and was shown to have actions opposite to that of ghrelin. Thus, if specific receptors for these peptides are present in adipocytes one can infer that it is likely that they may have a direct effect on adipose tissue function.

The work presented in this thesis has demonstrated that genes encoding the receptors for ghrelin (GHS-R), obestatin (GPR39-putative), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) are expressed in the main WAT depots of mice and humans, and indeed are expressed in cell systems of both mouse and human adipocytes. Moreover, the receptor protein was detected in adipose tissues and in differentiated adipocytes by western blotting. In addition, receptor mRNA was detected in subcutaneous adipose tissue of obese human subjects. Importantly, while GPR39 gene expression was found in subcutaneous fat of obese subjects no expression was evident for GPR39 in lean subjects.

The work has suggested that both GIP and ghrelin serve as regulatory agents that may have a role in the inflammatory response in human adipocytes. In addition, treatment with different gut hormones like the incretins, GIP and GLP-1, or ghrelin has a selective effect on the production of a number of adipokines in human adipocytes.

This might constitute an important element in the pathogenesis of obesity. Moreover, obestatin (1-23) and obestatin (11-23) may have regulatory effects on the release of key adipokines, such as adiponectin, IL-6, MCP-1 and in the case of obestatin (11-23), leptin.

Finally, 24 h treatment with GLP-1 and ghrelin in the absence or presence of insulin in fully differentiated SGBS adipocytes showed significant stimulatory effects on glucose uptake. Although it was not possible to identify the precise mechanism of this effect, it would *not* appear to be through induction of the insulin-sensitive glucose transporter, GLUT4.

In conclusion, these studies indicate that the receptors for gut hormones are expressed in WAT and are indeed present in differentiated adipocytes. They also suggest that in differentiated human adipocytes, the incretins, obestatin and ghrelin regulate the expression and release of key adipokines and thereby play a significant role in the cross-talk between the gut and adipose tissue.

CHAPTER 1
INTRODUCTION

1.1 Historical

The physiology, origin, chemistry, pharmacology and clinical aspects of gastrointestinal (GI) hormones, or gut hormones as they have been more succinctly labelled, has become one of the most rapidly expanding and interesting fields in contemporary endocrinology and gastroendocrinology. One of the more eloquent pictures of the ever growing field of gut hormones was painted by G.B.J Glass

" This territory is reminiscent of a subtropical forest full of proud and established trees, jungle-like bushes inter-woven with each other, beautiful flowers not yet classified in the Linnaen code, and also frail and weak growths struggling for survival. In this new territory the steps of the explorer are both risky and highly rewarding, but certainly full of excitement." (Glass, 1980)

The study of gut hormones as we now know them, dates back to the very beginning of last century. Bayliss and Starling published a paper entitled "On the causation of the so called peripheral reflex secretion of the pancreas." (Bayliss and Starling, 1902).
The " peripheral reflex" referred to in their paper was that propounded by Popielski (1901) who claimed that the secretions of the pancreas were under nervous control. However, with Balliss and Staeling's discovery of secretin the age of "endocrinology" had begun and any serious thoughts of the involvement of the central nervous system (CNS) in the secretions of the pancreas were set aside for decades.
In 1905 Edkins described the chemical mechanism of gastric secretion with the discovery of gastrin (Edkins, 1905). More than twenty years later it was discovered that yet another hormone contributed to the normal function of the gut, when it was shown that introduction of fat into the small intestine resulted in the secretion of a hormone which caused gall bladder contractions. This hormone was named cholecystokinin (CCK) (Ivy and Oldberg, 1928), and thus the entire workings of the gut were apparently explained by the actions of these three hormones; gastrin controlled gastric acid secretion, secretin that of pancreatic bicarbonate, and cholecystokinin-pancreozymin (as it became known) gall bladder contraction and pancreatic enzyme output. Even as late as 1970 it was argued that the hormonal regulation of the digestive tract and pancreas could be totally explained by these same three hormones (Grossman, 1970).

Although the fuse was lit in 1902 the explosion in interest and " understanding" in the working of the gut did not occur until sixty to seventy years later, for example, the amino acid sequence of gastrin was not determined until 1964 (Gregory *et al.,* 1964)
A wealth of published studies have supported the hypothesis that more than one peptide can be involved in the regulation of GI function, for instance gastric and pancreatic secretion, and GI motility, acting as stimulants or inhibitors. However, more recently researchers have turned their attention to possible roles for GI hormones outside the GI tract.

1.2 The Gut

The primary function of the gut is the uptake of water and nutrients. Most of the nutrient molecules present in food are large polymeric molecules that must be mechanically and enzymatically broken down into smaller molecules before they can be absorbed.
Digestion, absorption, and secretion take place in different components of the GI tract and specific functions tend to be localized to the main regions of the gut, namely the stomach, the small intestine, and the distal and proximal colon.
There is a continuous back and forth passage of information between the GI tract, and the brain, known collectively as the brain-gut axis. In the CNS, complex circuitries process incoming signals and control metabolically active organs such as the liver, skeletal muscle, and adipose tissue (Spiegelman and Flier, 2001; Horvath *et al*, 2004). These interrelated circuits can influence the functions, sensitivity, secretion, inflammatory responses and motility of the gut (Fig 1.1).
Many peptides are synthesised and released from the GI tract. Whilst their roles in the regulation of GI function have been known for some time, it is now evident that they also influence eating behaviour and thus can be potential anti-obesity targets. Several peptides which affect food intake have been identified including ghrelin, obestatin, incretins [glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)] and many other peptides. Several physiological factors may be involved in the development of obesity. These include altered adipose tissue metabolism, hormonal changes, and changes at sites in the brain that control satiety, particularly in the hypothalamus. There may be some kind of abnormal signal that affects adipose tissue metabolism and alters fuel partitioning, directing increased

storage in adipose tissue instead of use in muscle. This abnormal signal has not been identified. However, lipoprotein lipase has been found to be increased in obesity, and this enzyme can increase the storage of triglycerides in adipose tissue. The molecular mechanisms of specific gut hormones supporting effect on lipogenesis are widely unclear apart from an increase of the insulin receptor affinity in adipocytes (Hauner *et al.,* 1988 and Bassil *et al.,* 2007). Despite evidence for GIP effects on fat metabolism in humans, the role of specific gut hormones in the pathogenesis of human obesity and insulin resistance has not been investigated in detail. However, specific receptors for GIP have been identified on adipose tissue, studies have been inconclusive with regard to GLP-1 and nothing is known with regards to ghrelin and obestatin. A direct effect on adipose tissue is highly likely if specific receptors for these peptide hormones are present in adipose tissue.

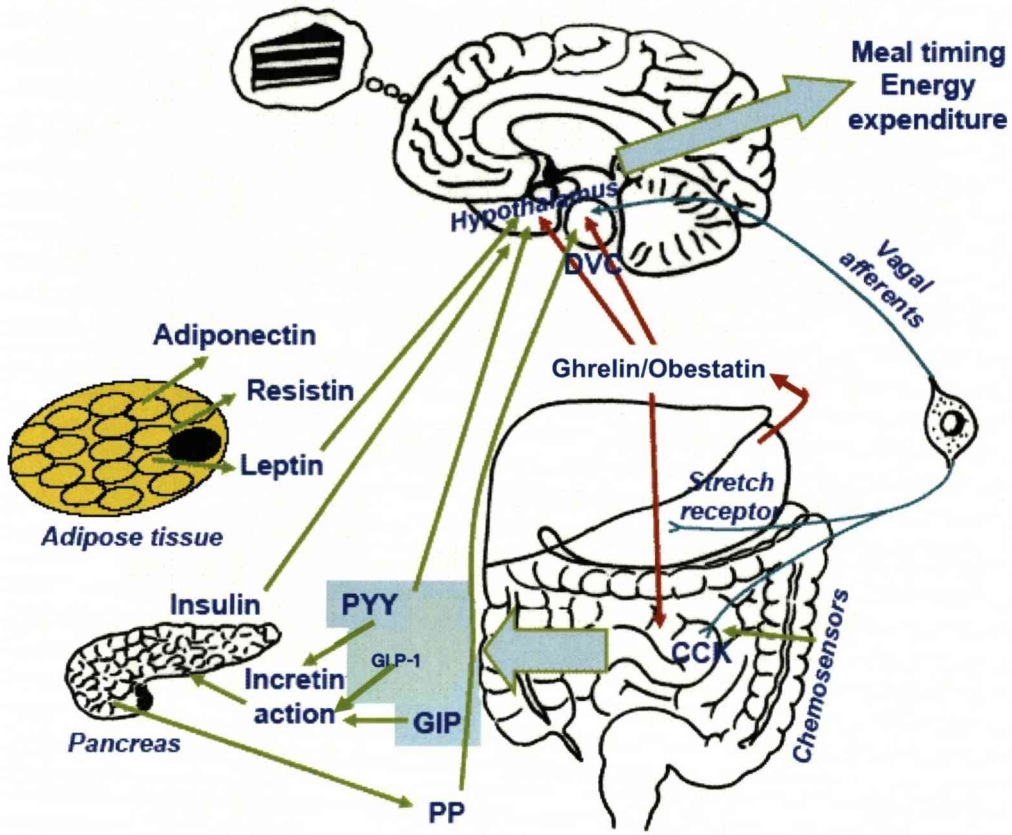
1.2.1 Incretins

The idea of regulation of the endocrine pancreas by signals from the gastrointestinal tract was proposed in 1929 by Zunz and La Barre. They termed this regulator an 'incretin'. Further support for this theory came with the observation by two independent groups in 1964 that glucose given orally led to a much greater increase in insulin than when an equivalent amount of glucose was given intravenously (Elrick *et al.,* 1964; McIntyre, Holdsworth and Turner, 1964). The explanation for this effect was that insulinotropic factors, incretins, were released from the gastrointestinal tract in response to nutrient (most notably glucose) absorption. A comparable experiment revealed that oral as opposed to intravenous amino acid loads caused a similar effect (Dupré *et al.,* 1968).

In order to be considered as incretin candidates peptides need to fulfill certain criteria: they must be released in response to ingestion of nutrients, especially glucose, and should reach postprandial concentrations *in vivo* that are of a sufficient magnitude to elicit an insulinotropic response at elevated glucose concentrations (Creutzfeldt, 1979).

Although several gut peptides and neurotransmitters exhibit incretin-like activity, evidence from experiments employing immunoneutralizing antisera, antagonists, and genetic loss-of function studies in mice suggests that GIP and GLP-1 are the dominant

Fig 1.1 The brain integrates peripheral signals relating food intake and energy balance



Adapted from Konturek et al., 2006

A schematic diagram of the brain integrating the peripheral signals produced by adipose tissue, pancreas and the gut, Feedbacks are related to the absorbed nutrients, gut peptides, adipose-derived protein factors and neural signals which balance the appetite, satiety and energy expenditure. PYY, peptide YY; CCK, Cholecystokinin; GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic peptide; DVC, dorsal vagal complex.

peptides involved in nutrient stimulated insulin secretion and account fully for the incretin effect (Fehmann *et al.*, 1989 and Nauck *et al.*, 1993).

1.2.1.1 Glucagon-like Peptide -1 (GLP-1)

Glucagon and the glucagon-like peptides are encoded in a much larger precursor known as proglucagon. Tissue-specific post translational processing leads to the production of the many forms of these peptides (Conlon, 1988; Drucker, 1990).

The sequence of human preproglucagon was determined (Bell *et al.,* 1983) and found to contain the sequence of glucagon and of two glucagon-like peptides referred to as glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). From examination of the gene sequence it was predicted that GLP-1 would contain 37 amino acids corresponding to proglucagon (Pederson *et al.*, 1975, Woods *et al.*, 2000). The presence of the glycine residue at the C-terminal suggested that the peptide is likely to be amidated by decarboxylation of glycine as is seen in other gastrointestinal hormones (Tatemoto and Mutt, 1980). This amidation is often found to be necessary for most hormones to be biologically active (Rehfeld *et al.,* 1980).
Thus two structures of GLP-1 were predicted corresponding to GLP-1 (1-37) and GLP-1 (1-36) amide. It was then demonstrated that GLP-1 undergoes further processing in the intestine whereby the 6 N-terminal amino acids are cleaved producing GLP-1 (7-37) or GLP-1 (7-36) amide (Mojsov *et al.,* 1986). Once this was discovered GLP-1 (7-36) amide was then isolated from the intestine of pigs (HoIst *et al.,* 1987), rats (Kreymann *et al.,* 1988) and humans (Ørskov *et al.,* 1989). The predominant form of GLP-1 in the intestine was shown to be the amidated form (Ørskov *et al.,* 1989).

GLP-1 has been found to be localised in the distal regions of the intestine, most notably the ileum and colon (Kreymann *et al.,* 1988). The endocrine cells in which GLP-1 is synthesised and secreted are also responsible for the secretion of enteroglucagon, GLP-2, glicentin (Ørskov *et al.,* 1986) and peptide YY (PYY) (Ali-Rachedi *et al.,* 1984) and are termed L cells. GLP-1 is also localised in the A-cells of the pancreas (Uttenthal *et al.,* 1985; Mojsov *et al.,* 1986; Ørskov *et al.,* 1986; Vaillant and Lund, 1986).

Glucose has been shown to stimulate the release of GLP-1 (7-36) amide (Kreymann *et al.,* 1987; Orskov *et al.,* 1991). Also a mixed meal results in increased secretion of GLP-1 (7-36) amide (Kreymann *et al.,* 1987). Studies have demonstrated increases in circulating concentrations of GLP-1 (7-36) amide following the ingestion of glucose, protein, fat or a mixed meal in humans (Elliott *et al.,* 1993; Ranganath *et al.,* 1996).

It is interesting to note that the postprandial peak in plasma GLP-1 (7-36) amide concentrations occurs within 30 minutes of the ingestion of the meal. Since the peptide is released from distal regions of the intestine it is unlikely that the nutrients have come into contact with the lumen or are absorbed here; thus the release may be stimulated by neural (Roberge and Brubaker, 1991) or hormonal signals from the upper gut (Brubaker, 1991). It has been established that GIP is not responsible for the stimulation of GLP-1 (7-36) amide secretion since it has been shown not to increase GLP-1 (7-36) amide concentrations when physiological concentrations of GIP was administred (Nauck *et al.,* 1991). The most documented action of GLP-1 (7-36) amide is its ability to stimulate the release of insulin during hyperglycaemia from the perfused pancreas (HoIst *et al.,* 1987; Mojsov, Weir and Habener, 1987). This action is discussed in greater detail in section 1.4.1.2.

GLP-1 (7-36) amide has been demonstrated to inhibit pentagastrin-stimulated gastric acid secretion in humans (O'Halloran *et al.,* 1990) suggesting a role for this peptide as an enterogastrone. GLP-1 (7-36) amide has been shown to stimulate the release of somatostatin from perfused pig pancreas (Ørskov, HoIst and Nielsen, 1988), perfused rat pancreas (Schmid *et al.,* 1990) and perfused rat stomach (Eissele, Koop and Arnold, 1990). Receptors for GLP-1 (7-37) have been discovered on a somatostatin secreting cell line (Fehmann and Habener, 1991). Receptors for GLP-1 (7-36) amide have also been found in the rat brain and lung (Kanse *et al.,* 1988). The presence of binding sites in the brain suggest that GLP-1 (7-36) amide may have a role as a neurotransmitter.

GLP-l receptor (GLP-1R) is a protein belonging to the family B of the seven transmembrane G-protein coupled receptors (Mayo *et al.,* 2003). Structure-activity studies suggest that the N-terminal comprises the activation domain and the C-terminal binding domain. GLP-1R is found on B- and D-cells of the pancreas,

parietal cells of the stomach, pylorus, lungs and the brain. The existence of receptors in the liver and skeletal muscle has not been confirmed.

The expression of the GLP-1R in adipocytes has been a matter of debate. Several studies have observed the expression of the GLP-1R *in vitro* by receptor-binding studies in human (Merida *et al.*, 1993), rat (Valverde *et al.*, 1993), and 3T3-L1 adipocytes (Egan *et al.*, 1994). However, in rat adipose tissue, others did not find GLP-1 receptors (Bullock *et al.*, 1996). Moreover, an alternative form of the receptor has been proposed (Montrose-Rafizadeh *et al.*, 1997). This alternative form may lower intracellular cAMP levels (Miki *et al.*, 1996), contrary to the conventional form, which stimulates formation of this second messenger.

1.2.1.2 Glucose-dependent insulinotropic polypeptide (GIP)

GIP is a 42 amino acid hormone originally discovered by its action of potent inhibition of gastric acid secretion (Brown *et al.,* 1970). It is localised in the duodenum and jejunum (Bloom and Polak, 1980) in cells termed K cells.

Secretion of GIP is stimulated by the ingestion of glucose in man (Cataland *et al.,* 1974) and in particular it is necessary for there to be active transport of glucose and other monosaccharides for this stimulation to occur (Sykes *et al.,* 1980). The other main stimulus for the secretion of GIP in man is ingestion of fat (Falko *et al.,* 1975) which is a more potent stimulant than glucose in man. Absorption of fat is a prerequisite for GIP secretion (Ebert and Creutzfeldt, 1980). Digestion of fat is necessary in order to elicit the response as GIP secretion is proportional to the quantity of long-chain fatty acids, which are the end product of triglyceride hydrolysis, in the gastrointestinal tract (Ross and Shaffer, 1981). It appears that chylomicron formation is a prerequisite for GIP secretion since blocking the formation of chylomicrons using a detergent (Tso *et al.,* 1981) results in inhibition of GIP release (Creutzfeldt and Ebert, 1988).

The first documented action of GIP was its ability to inhibit gastric acid secretion (Brown *et al.,* 1970), but this effect is weak in humans since increases in circulating concentrations of GIP caused by feeding were insufficient to cause any significant inhibition of acid secretion (Maxwell *et al.,* 1980). However, there is considerable evidence that GIP can inhibit the secretion of gastric acid and this action may be mediated by locally released somatostatin (Brown *et al.,* 1989).

The major biological activity of GIP is its potent stimulation of the secretion of insulin. It was observed that the infusion of GIP in humans together with an infusion of glucose resulted in potentiation of insulin release and improvement in glucose tolerance (Dupre *et al.,* 1973). This action of GIP is covered more extensively in section 1.4.1.1.

It has recently become apparent that GIP may have direct effects on lipid metabolism (Beck, 1989).

The GIP receptor (GIPR) belongs to the seven-transmembrane domain family of receptors. It is widely distributed in peripheral organs of animals (Usdin *et al.,* 1993). Although the GIP receptor is expressed in various regions of the brain, the actions of GIP in the central nervous system remain uncertain. Functional GIPR has also been identified in rat adipocytes (Yip *et al.,* 1998). In addition; studies reported that GIPR has alternative modes of action, both dependent and independent of cAMP, in rat adipocytes (Ehses *et al.,* 2002) and in non-adipose cell lines (Oben *et al.,* 1991, Beck *et al.,* 1983 and Beck *et al.,* 1988). Although GIPR is expressed in primary cultures of rat adipocytes (Yip *et al.,* 2000) and differentiated 3T3-L1 adipocytes (Kubota *et al.,* 1997), the functions of the hormones in fat cells, including whether it may affect adipokine production has not been established.

1.2.2 Ghrelin

Ghrelin is a 28 amino acid peptide that was originally identified as an endogenous ligand for the growth hormone secretagogue receptor (Horvath *et al.,* 2001). Ghrelin is acylated on serine-3, a modification observed for the first time in mammalian physiology, and this acylation appears to be necessary for its biological activity (Kojima *et al.*, 1999). Ghrelin is produced predominately by the stomach (Ariyasu *et al.*, 2001), but also in lesser amounts by the GI tract (Date *et al.*, 2000; Rindi *et al.*, 2002) kidney and in the hypothalamus and plays a role in the short-term regulation of energy homeostasis (Mori *et al.*, 2000; Gnanapavan *et al.*, 2002; Guan *et al.*, 1997). Recently, adminstration of ghrelin to rodents was shown to induce obesity by increasing food intake and reducing fat utilization (Kojima *et al.*, 1999). In human studies of ghrelin effects on growth hormone (GH) release, feelings of hunger were noted as a side effect in a majority of the test subjects (Arvat *et al.*, 2000). Serum ghrelin is reduced in obese humans and following acute overfeeding (Kim *et al.*, 2003). Circulating ghrelin is increased with fasting in humans. Ghrelin regulates food

intake by binding specific receptors in the hypothalamus and activating well-characterized arcuate nucleus neurons, which produce neuropeptide Y (NPY) and agouti related peptide (AGRP) to stimulate feeding (Morton *et al.*, 2001). Ghrelin has also been reported to act on other signalling pathways in the hypothalamus and much work is underway to fully understand the signalling pathways and role of ghrelin in the regulation of food intake (Nakazato *et al.*, 2001). Ghrelin receptor, or GHS-R, is a typical G-protein coupled receptors (GPCR) with seven transmembrane domains (7-TM) (Howard *et al.*, 1996; McKee *et al.*, 1997; Smith *et al.*, 1999). Two distinct ghrelin receptor cDNAs have been isolated (Howard *et al.*, 1996). The first, GHS-R type la, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor, and another GHS-R type 1b, is produced by an alternative splicing mechanism (Howard *et al.*, 1996). The ghrelin receptor occurs in several CNS sites such as arcuate and ventromedial nuclei (Howard *et al.*, 1996), as well as in many peripheral regions including heart, lung, stomach, kidney (Kojima *et al.*, 2001) and adipose tissue (Kim *et al.*, 2004), indicating that ghrelin is likely to have multiple functions in these tissues.

1.2.3 Obestatin

Obestatin is a 23 amino acid peptide produced in the stomach that appears to be involved in the regulation of energy balance (Zhang *et al.*, 2005). Obestatin is transcribed from the preproghrelin gene and C-terminal amidation is a prerequisite for biological activity (Zhang *et al.*, 2005). Several of the actions of obestatin appear to oppose the actions of ghrelin (Szentirmai and Krueger 2006; Tremblay *et al.*, 2007). Most notably, obestatin appears to be a potent appetite-suppressant causing reductions in food intake, body weight and jejunal contraction in rodents (Zhang *et al.*, 2005). Also, recent information indicates that obestatin reduces fluid intake by altering of thirst mechanisms within the brain (Samson *et al.*, 2007).

The G-protein coupled receptor, GPR39, has been proposed to be the target receptor of obestatin (Zhang *et al.*, 2005). However, this has not been confirmed by other studies (Holst *et al.*, 2007; Lauwers *et al.*, 2006). One study showed that the GPR39 is present in many regions of the brain including the pituitary gland, hypothalamus and cerebral cortex (Zhang *et al.*, 2005). However, this conflicts with recent evidence of the absence of both GPR39 in the hypothalamus (Jackson *et al.*, 2006) and obestatin

in the brain (Pan *et al.,* 2006). More puzzlingly, it would appear that obestatin is not transported across the blood–brain barrier (Pan *et al.,* 2006). Although some studies agreed that GPR39 is expressed in various regions of the brain, the actions of the obestatin peptide in the central nervous system remain unconfirmed. GPR39 appears to be strongly expressed in the jejunum, duodenum, stomach, ileum and liver, but has also been found to a lesser extent in the pancreas (Zhang *et al.,* 2005). Although the presence of GPR39 in pancreatic tissue has not been confirmed by others (Jackson *et al.,* 2006), significant levels of obestatin immunoreactivity were recently reported in perinatal rat pancreas (Chanoine *et al.,* 2006). Furthermore, obestatin immunoreactivity positively correlated with insulin concentrations, and since acylated ghrelin, which is also found in the pancreas, inhibits insulin secretion, it has been suggested that obestatin may potentiate insulin release (Chanoine *et al.,* 2006).

1.3 ROLE OF GASTROINTESTINAL HORMONES IN OBESITY

Treatment of obesity is likely to be eventually revolutionised by greater understanding of the disease. Knowledge in this field is still limited to a few "culprit factors" that might be involved in the perpetuation of the disease, while less is known about the precise role of the individual factors in the causation or initiation of obesity. Discovery of various peptides including, ghrelin, leptin and obestatin has given obesity research a boost, opening up areas for further studies.

1.3.1 Obesity

Obesity has become a major public health issue in western countries, and is starting to spread to developing countries. According to the figures provided by World Health Organization (WHO), globally, more than 1 billion adults are overweight; among them, at least 300 million are clinically obese. In England, a recent figure has shown that about two-thirds of its population are overweight or obese (House of Commons Health Committee, 2003). Even more seriously, the "epidemic" of obesity has rapidly spread to children and adolescents. The high proportion of obesity in a population not only reduces the quality of life since obese individuals are more likely to develop type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, sleeping apnoea,

osteoarthritis and certain cancers, but also results in a high economic burden to societies.

1.3.1.1 Prevalence and causes of Obesity

Obesity is caused by an imbalance between energy intake and energy expenditure. More precisely, obesity can only develop when energy intake is in excess of energy expenditure (Trayhurn, 2005). Generally, overweight and obesity are defined by a criterition based on calculation of the body mass index (BMI), which is assessed by dividing an individual's body weight measured in kilograms by his/her height in metres squared (kg/m^2). The accepted classification ranges are; BMI> 17.5 kg/m^2 and < 24.9 kg/m^2 as normal, ≥25-29.9 kg/m^2 as overweight, > 30 kg/m^2 as obesity and > 40 kg/m^2 as morbid obesity. However, in some circumstances, BMI ≥ 25 kg/m^2 cannot necessarily be considered as overweight or obesity, for instance, in athletes and muscular individuals, who have more skeletal muscle than normal subjects; muscle mass is heavier than fat mass, and therefore, the enhanced proportion of muscle mass leads to an increased body weight. Another shortcoming of BMI is that it is unable to illuminate fat distribution in the body; in other words, BMI cannot distinguish the precise location of the extra body fat. Based on the previous approaches, a high accumulation of visceral fat is thought to be more highly linked to obesity-associated complications than subcutaneous fat (Bray, 2004). Thus, additional methods, including waist:hip ratio, computed tomography (CT) and magnetic resonance imaging (MRI), can be supportively used to diagnose obesity together with BMI.

Obesity is the consequence of unbalanced food intake and energy expenditure. It is believed that obesity is trigged by an interplay of genetic and environmental risk factors. Although a monogenic mutation occasionally can be traced in some obese families, it is more likely that multiple genes are involved in the underlying development of obesity in humans. In addition, environmental risk factors are major contributors to the pandemic of obesity. Foods with high fat are supplied routinely in the Western diet. Reduced physical activity also has a key role in weight gain. The sedentary life style markedly decreases metabolic rate. Overall, increased food intake and reduced energy expenditure results in extra energy stored as fat in white adipose

tissue (WAT).

1.3.1.2 Obesity associated diseases

Obesity is one of the components that is part of the metabolic syndrome, diagnosis of which requires the presence of at least three out of the following five criteria: impaired insulin sensitivity, hyperglycaemia, dyslipidaemia (elevated blood triacyglycerols with depressed HDL-cholesterol), abdominal obesity and hypertension. The syndrome represents a group of metabolic and vascular disorders that increases the risk of developing type 2 diabetes and cardiovascular disease (Shaw *et al.*, 2005).

Obesity itself is linked to a greater than 10-fold higher risk of developing type II diabetes (previously called non-insulin-dependent diabetes), which results from an inability of the tissues in the body to respond appropriately to insulin produced by the pancreas (WHO, 2002). This risk of developing the condition increases with the degree of obesity (Diabetes UK, 2005).

Cardiovascular disease is also strongly associated with obesity, with higher rates of coronary heart disease, hypertension, atherosclerosis and dyslipidaemia present in the obese (Shaw *et al.,* 2005). A meta-analysis that collated data from 26 separate studies found that coronary heart disease is the most common cause of premature death in obese people (McGee, 2005). A 16-year prospective study in the USA linked obesity with an increased risk of death from cancer, with a higher incidence of certain cancers, including breast, ovarian and prostate occurring in the obese (Calle *et al.,* 2003), and obesity is also associated with a greater risk of developing certain orthopaedic conditions such as osteoarthritis, respiratory diseases (including sleep apnoea) and a range of renal disorders (Formiguera & Canton, 2004).

The location of fat stores is also important in determining the susceptibility to obesity associated diseases, due to functional differences between visceral and subcutaneous fat (Wajchenberg, 2000; Atzmon *et al.,* 2002). Increased adiposity in the upper part of the body, corresponding to visceral or abdominal fat accumulation, has been linked to the development of glucose intolerance, hyperinsulinaemia,

hypertriglyceridaemia, hypertension and sleep apnoea (Wajchenberg, 2000; Vgontzas *et al.,* 2003; Bray, 2004). Contrastingly, those with gluteal-femoral or peripheral adiposity (lower body obesity, or female-type fat distribution), have been found to be less susceptible to these morbidities (Wajchenberg, 2000).

1.3.1.3 Obesity and white adipose tissue

Extra energy is largely stored as lipid within adipocytes. The enlarged size of adipocytes in obesity is an initial compensation to buffer the increased content of triacylglycerol (TAG) in cells. However, in the long term, the extra lipid may exceed the storage capacity of adipocytes, which results in the differentiation of preadipocytes in the stromal vascular (SV) fraction into adipocytes and finally increases total adipocyte numbers (Spiegelman & Flier, 1996).

Obesity has emerged as a low grade inflammatory state. According to the description given in section 1.6, overproduction of several cytokines and acute-phase proteins by WAT, such as Tumour necrosis factor-α (TNFα), plasminogen activator inhibitor-l (PAI-1), monocyte chemoattractant protein-l (MCP-l) and Interleukin-6 (IL-6), together with the inhibition of adiponectin, an anti-inflammatory adipokine, is proposed to be an important feature in obesity (Trayhurn & Wood, 2004). It is still unclear whether adipocytes or the SV fraction in WAT has a more prominent role in the secretion of these proinflammatory factors (Fain *et al.,* 2004). A remodelling of WAT by cross-talk between these two fractions is likely to mediate the low-grade inflammatory response in obesity. Evidence indicates that the infiltration of macrophages into WAT is an important phenomenon in obesity (Weisberg *et al.,* 2003; Xu *et al.,* 2003). These recruited macrophages derived from bone marrow appear to be a response to the increased production of MCP-1 by both adipocytes and the SV fraction in WAT and contribute to the development of obesity-associated systemic inflammation (Weisberg *et al.,* 2003; Xu *et al.,* 2003). Recently, a study has shown that in WAT, the majority of macrophages are located around dead adipocytes scavenging debris by phagocytosis (Cinti, 2005).

Enhanced lipolytic activity is another characteristic aspect of obesity and infection (Wellen & Hotamisligil, 2005). The activation of lipolysis in WAT is likely to be partly due to the systemic inflammatory response in obesity as TNF-α and leptin stimulate lipolysis locally in adipocytes (Kawakami *et al.,* 1987; Ryden *et al.,* 2004;

Ahima, 2006). In addition, the increased output of free fatty acids (FFA) leads to the accumulation of lipid droplets in other metabolic organs, such as liver, skeletal muscle, heart and pancreas, an event termed lipotoxicity (Lelliott & Vidal-Puig, 2004).

Overall, the elevated levels of inflammatory markers and dyslipidaemia are involved in the development of a low-grade systemic inflammation in obesity. This directly or indirectly leads to insulin resistance and disorders of metabolic activity, which eventually result in the complications of obesity, such as type 2 diabetes and atherosclerosis.

1.3.2 GLP-1 and Obesity

GLP-1 decreases appetite in healthy (Flint *et al.,* 1998), obese (Näslund *et al.,* 1999) and diabetic humans (Gutzwiller *et al.,*1999a). Whether this is mediated through direct action on central pathways or predominantly through peripheral actions (gastric distension due to delay in gastric emptying) remains to be clarified. The fact that GLP-1 reduces appetite even in fasted patients (Gutzwiller *et al.,* 1999b) argues for a significant role for central mechanisms. Yet, the fact that the half-life of active GLP-1 is less than 2 minutes makes it likely that peripheral mechanisms such as delayed gastric emptying play a significant role.

One study showed that intraventricular administration of GLP-1 in fasted rats inhibits food intake (Tang-Christensen *et al;* 1996). This inhibition in food intake seemed to be a short-term effect rather than a long-term meal regulation effect as shown by 4-day infusion studies in rats (Donahey *et al.,* 1998). The inhibitory effects of GLP-1 on food intake may involve direct or indirect effects on hypothalamic feeding centres, and may be mediated in part through inhibition of gastrointestinal motility. The actions of GLP-1 on food intake may also be due in part to induction of a conditioned taste aversion response and activation of CNS aversive signaling pathways (Kinzig, *et al.,* 2002, Seeley *et al.,* 2000).

Exendin-4 is a novel 39-amino acid peptide isolated from the venom of the Gila monster *Heloderma suspectum.* It shares 53% sequence homology with GLP-17-36 amide and interacts with the same membrane receptor. Exendin-4 has a significantly greater half-life in human serum compared to GLP-1. Exendin-4 has recently been shown to significantly lower fasting plasma glucose, delay gastric emptying, and

reduce food intake in healthy human volunteers (Edwards *et al.,* 2001). Exendin-4 may potentially be useful in the future in the treatment diabetes and obesity.

1.3.3 GIP and Obesity

A recent study in GIPR-deficient mice has linked GIP to the development of obesity through an effect on adipose tissue triggered by overnutrition (Miyawaki *et al.,* 2002). GIP receptor knock-out prevents the development of obesity and insulin resistance in *ob/ob* mice despite high fat feeding. Despite the decreased insulin response and hyperglycaemia the feeding behaviour in these rodents was unaffected. Obese subjects have increased fasting concentrations of GIP as well as an early-enhanced postprandial GIP response compared to lean subjects (Vilsbøll *et al.,* 2003). In fact, GIP receptor antagonists may have a part to play in future obesity treatment (Gault *et al.,* 2003), although the implications of GIP-blockade on carbohydrate and lipid metabolism might well restrict its evolution into a therapeutic agent for obesity in the near future.

1.3.4 Ghrelin and Obesity

Ghrelin is produced primarily in gastrointestinal organs in response to hunger and starvation, and circulates in the blood, serving to signal the central nervous system to stimulate feeding.

Recent identification of appetite-regulating humoral factors reveals regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (Neary *et al.,* 2004; Ukkola *et al.,* 2004). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain (Friedman, 2002); Hunger signals from peripheral tissues, however, had remained unidentified until the recent discovery of ghrelin. When ghrelin is injected into the cerebral ventricles of rats, their food intake is potently stimulated (Kamegai, *et al.,* 2001; Nakazato, *et al.,* 2001; Tschop *et al.,* 2000).

Among all discovered orexigenic peptide, ghrelin has been found to be the most powerful. Chronic intracerebroventricular injection of ghrelin increases cumulative food intake and decreases energy expenditure, resulting in body weight gain.

Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight. Not only intracerebroventricular injection, but also intravenous

and subcutaneous injection of ghrelin has been shown to increase food intake (Nakazato, *et al.,* 2001;Tschop *et al.,* 2000; Wren, *et al.,* 2001).

1.3.5 Obestatin and Obesity

Initial studies showed that obestatin has opposing effects to ghrelin. When given centrally and peripherally to mice, it resulted in decreased food intake and suppressed gastric emptying. Given chronically over 8 days, obestatin also decreased weight gain in mice, as compared to vehicle (Zhang *et al.,* 2005). Unlike with ghrelin, fasting rodents for 48 h and then re-feeding has no effect on obestatin levels (Zhang *et al.,* 2005). Since the original publications two groups have partially confirmed the effects of obestatin on food intake and gastric emptying, (Bresciani *et al.,* 2006; Moechars *et al.,* 2006), but two other groups have failed to replicate these findings (Nogueiras *et al.,* 2007; Seoane, *et al.,* 2006). The significance of obestatin in the short-term regulation of food intake therefore remains uncertain.

1.4 ROLE OF GASTROINTESTINAL HORMONES IN THE REGULATION OF INSULIN SECRETION

1.4.1 Enteroinsular axis

The term 'enteroinsular axis' was suggested by Unger and Eisentraut (1969) to describe the regulation of endocrine secretion from the pancreas. This included regulation by hormones, neural factors and substrate stimulation originating from the alimentary tract and the pancreatic islets, which promotes insulin release in response to feeding. The pancreas has an abundant supply of autonomic nerve fibres which enter the organ with the arteries (Woods and Porte, 1974), suggesting that nervous control of pancreatic secretions is important. Cholinergic, adrenergic and peptidergic neurons have all been demonstrated.

The role of cholinergic nerves in the control of endocrine secretion from the pancreas appears to be in the early (cephalic) insulin response to a meal and not associated with the absorption of nutrients. It has also been proposed that cholinergic control may be involved in some models of animal obesity and in the regulation of basal and

postprandial insulin secretion (Ahren *et al.,* 1986).

The role of adrenergic neurones in the control of endocrine secretion appears to be related to stress states. In this case there is dual control; α-adrenergic activity inhibits insulin secretion and β-adrenergic activity leads to stimulation of insulin secretion (Ahren, Taborsky and Porte, 1986). These opposing adrenergic influences make the net effect on insulin secretion very difficult to assess.

There are many peptides that have been shown to be present in nerve terminals in the pancreas, which may be involved in peptidergic regulation of pancreatic endocrine secretion. These include vasoactive intestinal peptide (VIP), cholecystokinin, gastrin releasing polypeptide, galanin, neuropeptide Y (NPY), calcitonin gene-related peptide, substance P and enkephalin.

Observation of a change in the circulating concentrations of insulin is not necessarily a result of variation in the rate of its secretion, but may occur in response to modification of the rate of hepatic extraction of this hormone. Creutzfeldt and Ebert (1985) proposed that the alteration in the rate of hepatic extraction of insulin plays a major part in the incretin effect. It is possible to estimate the rate of insulin secretion by measuring the plasma concentration of C-peptide. This peptide is co-secreted with insulin in equimolar amounts, but unlike insulin is not subject to hepatic extraction and so can be used to quantify insulin secretion rates.

Intravenous glucose was found to be associated with higher rates of hepatic insulin extraction than oral glucose (Nauck *et al.,* 1986; Shuster *et al.,* 1988) and it was suggested that this difference may be due to the action of gastrointestinal hormones or neural signals released in response to the absorption of nutrients.

1.4.1.1 Role of GIP in the enteroinsular axis

Soon after the isolation of GIP, its insulinotropic actions were demonstrated in humans (Dupre *et al.,* 1973; Anderson *et al.,* 1979; Elahi *et al.,* 1979). When human subjects were given intravenous porcine GIP simultaneously with intravenous glucose there was a greater insulin response and improved glucose tolerance compared with glucose infusion alone (Dupre *et al.,* 1973). This effect could be reduced in rats by the infusion of anti-GIP antiserum (HoIst, Lauritsen and Moody, 1980; Ebert and Creutzfeldt, 1982).

The potent ability of GIP to stimulate insulin secretion was demonstrated to be

glucose-dependent in the perfused rat pancreas (Pederson and Brown, 1978). This was confirmed in humans where ingestion of an oral fat load stimulated the release of GIP but not insulin. When the fat load was ingested together with intravenous administration of glucose the insulin response was greater than when the infusion of glucose was given alone (Crockett *et al.,* 1976). This observation confirmed the insulinotropic action of GIP to be glucose-dependent. Also, when the fat load was ingested during the infusion of intravenous glucose the GIP response was smaller than that observed when the fat meal was ingested alone suggesting feedback inhibition by insulin (Crockett *et al.,* 1976).

Since the insulinotropic effect was not totally abolished by the administration of anti GIP antiserum in rats it was thought likely that GIP is not the only incretin (HoIst *et al.,* 1980; Ebert and Creutzfeldt, 1982).

1.4.1.2 Role of GLP-1 in the enteroinsular axis

GLP-1 (7-36) amide was demonstrated to be a physiological incretin, possibly more powerful than GIP (Kreymann *et al.,* 1987).

GLP-1 (7-36) amide was considered to be a gastrointestinal hormone of physiological significance following the discovery of specific binding sites for GLP-1 (7-36) amide on pancreatic B-cells (Goke and Conlon, 1988). It was also confirmed that GLP-1 (7-36) amide had potent insulinotropic effects (Ørskov *et al.,* 1988; Shima *et al.,* 1988). The insulinotropic effect of GLP-1 (7-36) amide is glucose-dependent as demonstrated from studies using perfused porcine pancreas (HoIst *et al.,* 1987) and perfused rat pancreas (Mojsov *et al.,* 1987).

1.4.1.3 Role of other gastrointestinal hormones in the enteroinsular axis

Cholecystokinin has been shown to stimulate the release of insulin from the endocrine pancreas (Unger *et al.,* 1967; Dupré *et al.,* 1969).

The identification of the pancreatic ghrelin-expressing cells is a matter of controversy, and the role of ghrelin in insulin secretion is likewise under debate. Ghrelin has been shown to inhibit insulin secretion in some experiments and stimulate insulin release in others (Adeghate and Ponery 2002, Broglio *et al.,* 2001, Lee *et al.,* 2002, Reimer *et al.* 2003).

These discrepancies may be due to species differences and/or experimental design. Plasma ghrelin and insulin concentrations are affected by blood glucose level; high glucose suppresses ghrelin secretion and stimulates insulin secretion, thus the glucose level in experiments may be important. Date *et al.* 2002 reported that ghrelin stimulates insulin release in the presence of high levels of glucose that could release insulin from cultured islet cells. In contrast, ghrelin had no effect on insulin release in the context of a basal level of glucose.

The recent discovery of obestatin in 2005 means that many of its biological actions remain to be identified, including whether this peptide influences *in vivo/vitro* glucose homeostasis or insulin secretion. Since ghrelin raises blood glucose and lowers insulin secretion, a number of studies are still investigating whether obestatin administration might exert opposite effects on glucose homeostasis and insulin secretion. However Green *et al.,* (2007) observed that obestatin peptide has direct and indirect effects on food intake, but no direct actions on glucose homeostasis or insulin secretion were indicated in mice.

1.5 WHITE ADIPOSE TISSUE

White adipose tissue (WAT) is very important to maintain energy homeostasis. WAT contains adipocytes, which are filled with a large lipid droplet that is surrounded by cytoplasm. The nucleus is always flattened and is found on the periphery. The lipid is stored in a semi-liquid state, and is composed primarily of triacylglycerols. WAT was known to have a simple structure that stores large amounts of triacylglycerols (TAGs) when there was extra energy, and delivered fatty acids to other tissues and organs. Thus, the size of the adipose tissue stores increases in periods of positive energy balance and declines when energy expenditure is in excess of intake (Trayhurn and Beattie, 2001). The SNS is the major regulator of lipolytic activity in WAT, although there is also regulation by nutrient and hormonal signals, including glucose and insulin (Ahima & Filer, 2000; Bartness *et al.,* 2001)

WAT is also an insulator, which can help in regulating the body temperature of mammals (for example, blubber in whales) (Klaus, 2001). Adipose tissue also affords a degree of mechanical protection when interposed between bony structures and soft tissue (Klaus, 2001). WAT secretes certain hormones that affect energy homeostasis,

which includes inflammatory development for example cytokines, regulators of lipoprotein metabolism and growth factors, among others (Mohamed-Ali *et al.*, 1998). Moreover, since the discovery of leptin in 1994 (Zhang *et al.,* 1994), there has been an appreciation of WAT as an endocrine organ, and these endocrine functions of adipose tissue will be described in section 1.6.

1.5.1 Anatomy of WAT

WAT contains mature adipocytes and the stromal-vascular (SV) fraction, which comprises macrophages, fibroblasts, vascular endothelial cells and preadipocytes (Hausman, 1985). Mature adipocytes account for about one-third to 50% of the cellular components in WAT (Avram *et al.,* 2005). A single white adipocyte has a spherical shape with one large 'unilocular' lipid droplet in the cytosol, which pushes the cytoplasm and the nucleus into a thin rim (Cinti, 2002). The size of white adipocytes normally ranges from 30 to 70 μM in diameter (Napolitano, 1963).

Adipocytes will become hypertrophied in an obese state; an enlarged fat cell can be greater than 150 μM in diameter (Chen *et al.,* 2006). Increased adipocyte numbers, known as hyperplasia, may also take place in morbid or childhood obesity due to the differentiation of preadipocytes into mature adipocytes (Spiegelman & Flier, 1996). In addition, it has been observed that the number of macrophages is markedly increased in WAT in obesity (Weisberg *et al.,* 2003; Cinti *et al.,* 2005). In contrast, reduced size of adipocytes has been seen in WAT in cancer cachexia; adipocytes lose their spherical shape and the lipid content is depleted (Bing *et al.,* 2006).

Histologically, adipocytes are not isolated since they are adjacent to capillaries (Cinti *et al.,* 2005). Although WAT is not as highly innervated as brown fat, the tissue is innervated by nerve fibres from the sympathetic nervous system (SNS) (Bartness & Bamshad, 1998). In view of this, the autonomic nervous system may also play fundamental roles in modulating the metabolism of WAT, particularly lipolysis and/or lipogenesis (Romijn & Fliers, 2005).

WAT is generally divided into subcutaneous and visceral depots according to the different location and function (Avram *et al.,* 2005). In rodents, subcutaneous fat depots are mainly located at the base of the forelimbs and hind legs (Cinti, 2005), whereas visceral depots are present in the thorax and abdominal cavity, which are further categorised as perigonadal, perirenal and mesenteric depots (Cinti, 2005). In

humans, subcutaneous depots are the predominant fat and account for up to 80% of total body fat (Arner, 1997). Subcutaneous fat pads are located beneath the layer of skin and play an important role in preventing heat loss in a cold environment. Visceral fat pads can be subdivided into intraperitoneal and retroperitoneal WAT according to the anatomy (Smith *et al.,* 2001). Intraperitoneal WAT includes omental and mesenteric fat depots, which account for the majority of the visceral fat (Smith *et al.,* 2001). Studies suggest that the highest metabolic activities occur in visceral fat and visceral obesity is closely linked to metabolic disorders (Arner, 1997).
Additionally, expression of several adipose tissue-derived proteins varies between subcutaneous and visceral WAT (Gesta *et al.,* 2006), which may partly explain the presence of unequal development of adipocytes between the two depots; moreover, it may also be the molecular mechanism underlining the difference in pathogenetic contribution between visceral fat and subcutaneous fat in obesity.

1.5.2 Development of adipose tissue

The development of WAT begins in embryos in humans (Poissonnet *et al.,* 1988). Foetal fat is minimal in the first two trimesters of pregnancy, but starts to substantially accumulate in the third trimester (Ziegler *et al.,* 1976). The proportion of fat accounts for about 15% of total body mass after birth in humans (Fomon *et al.,* 1982).

1.5.3 Adipogenesis

Studies of the development of white adipose tissue focus on adipogenesis, a process by which the multipotent mesenchymal stem cells differentiate into mature adipocytes under appropriate stimuli (Rosen & Spiegelman, 2000). Convincing evidence suggests that adipogenesis occurs both in the foetus and after birth, and continuously throughout life (Rosen & Spiegelman, 2000). Adipogenesis includes two stages: firstly, the multipotent mesenchymal stem cells are induced to the preadipocyte lineage; secondly, adipogenic signals further trigger the differentiation of fibroblastic-like preadipocytes into spherical shaped adipocytes with lipid accumulation inside the cells (Gregoire *et al.,* 1998). In addition to morphological alterations, adipogenesis is also associated with biochemical changes, such as the expression of specific genes in the early stage of adipocyte differentiation, which in

turn are used as markers to reflect the differentiation (Ailhaud, 1996).

The molecular mechanisms which determine how the mesenchymal precursor cells undergo the transformation to the adipocyte lineage and further differentiation of preadipocytes into mature adipocytes have not been fully established. However, based on previous studies, a key development of adipogenesis research is the identification of two major transcription factors, the CCAAT/enhancer binding protein α (C/EBP α) and the peroxisome proliferator-activated receptor γ (PPARγ) (Gregoire *et al.,* 1998). It has been demonstrated that *in vitro* and *in vivo* activation of these two transcription factors play a fundamental role in mediating adipocyte differentiation and is necessary to initiate the transduction of differential signalling cascades. C/EBPα is a family member of the basic-leucine zipper class of transcription factors and has predominant roles in the induction of preadipocytes and fat accumulation (Rosen & Spiegelman, 2000). PPARγ has two isoforms, PPARγ1 and PPARγ2, due to differential splicing near the 5'end in the gene encoded sequence (Fajas *et al.,* 1997). PPARγ2 is abundantly expressed in adipose tissue, and is the most likely isoform influencing adipogenesis in the tissue (Fajas *et al.,* 1997). It has been shown that incubation of preadipocytes with PPARγ ligands leads to a remarkable stimulation of adipogenesis (Gurnell *et al.,* 2003). In contrast, depletion of the PPARγ receptor encoded gene in mouse embryos results in the death of the embryo (Barak *et al.,* 1999), suggesting that the PPARγ receptor is necessary for the development and survival of embryos. However, a functionally mutated PPARγ gene causes a significant reduction of fat mass and lipodystrophy in mice (Rosen & Spiegelman, 2000). In addition to stimulating adipogenesis, the PPARγ receptor has roles in the modulation of insulin sensitivity, hypertension and dyslipidaemia (Gurnell *et al.,* 2003).

1.5.4 Apoptosis of adipocytes

Apoptosis is the self-regulated deletion of a dysfunctional cell or 'programmed cell death'. It involves a large number of molecules and is governed by several integrated pathways. Although body weight loss primarily comes from the reduced size of adipocytes and not the number of cells, apoptosis is still present in adipocytes and is correlated with the depletion of fat cell numbers in WAT during weight loss (Gullicksen *et al.,* 2003); for instance, apoptosis of adipocytes has been observed in

WAT of cancer cachectic patients and human immunodeficiency virus (HIV) patients treated with protease inhibitors (Prins *et al.,* 1994; Domingo *et al.,* 1999). Leptin and TNF-α, two adipocyte-derived protein factors, have been shown to link with adipocyte programmed cell death (Gullicksen *et al.,* 2003; Warne, 2003).

1.5.5 Studies of WAT

1.5.5.1 *In vivo* study of WAT

In vivo investigation is a very powerful tool to explore the specific environmental effects or the effects of foods on metabolism. For example, the influence of a certain chemical compound or the activity of a pharmacological drug can be examined by being given to live animals through oral administration or direct injections. *In vivo* knock out of a specific gene in a live animal is an extremely useful method to clarify the functions of the encoded protein. However, the major shortcoming of an *in vivo* study is that it is unlikely to precisely identify the exact molecules affected by the treatment. Thus, at some point, *in vitro* studies and particularly using cultured cells are a key way to examine the potential signalling pathways and the downstream molecules in response to a certain treatment.

1.5.5.2 *In vitro* cell culture models to study adipocytes

Currently, at least three types of *in vitro* culture models have been employed for studying adipocytes: i) mature adipocytes isolated from white fat pad, ii) preadipocyte primary culture, and iii) preadipocyte clonal cell lines (Gregoire *et al.,* 1998). These culture models provide effective tools for studying adipogenesis and the functions of adipocytes.

1.5.5.2.a Mature adipocytes isolated from fat pads

Collagenase digestion is a straightforward way to separate mature adipocytes directly from a fresh fat pad. The isolated mature adipocytes are seeded and maintained in media with essential nutrients or salt solution. Mature adipocytes are able to be

maintained for a period of time but normally no longer than 24 h. It is thought that mature adipocytes isolated by this method are closer to *in vivo* adipocytes since they are immediately separated from WAT of an intact animal. However, caution should be exercised in data analysis since excision and mincing of tissues are disruptive procedures that may influence the outcome of the experimental results. For example, induced expression of tumour necrosis factor-α (TNF-α) and hypoxiainduced factor-lα (HIF-lα) was observed in mature adipocytes prepared from minced adipose tissue (Gesta *et al.,* 2003; Ruan *et al.,* 2003).

1.5.5.2.b Preadipocyte primary culture

Primary culture is a method to obtain adipocytes from the isolated preadipocytes in the SV fraction of fresh WAT. The cultured preadipocytes are maintained in a medium with all the necessary nutrients. Following confluence, preadipocytes are then induced to differentiate into adipocytes by exposure to a medium containing components of an adipogenic cocktail. In comparison with the clonal aneuploid cell lines, it reflects the *in vivo* situation more closely because primary preadipocytes are diploid (Gregoire *et al.,* 1998). Another key advantage of preadipocyte primary culture is that preadipocytes can be derived from a variety of fat depots, such as subcutaneous and visceral. This property raises the possibility of analysing the difference between subcutaneous and visceral fat depots in term of their capacity to differentiate and differences in biochemical activity. However, one problem of primary preadipocyte culture is the purity of this culture system since there may be trace amounts of other cell types in the SV fraction, such as fibroblasts. Another shortcoming is that primary culture needs a large amount of fresh adipose tissue to collect sufficient preadipocytes. Moreover, primary culture is unable to be repeated since the preadipocytes have a limited capability to undergo passage; in other words, the preadipocytes lose their ability to be induced into adipocytes after culturing for a couple of generations.

Zen-Bio cells are a commercially available primary culture derived from the preadipocytes in the SV fraction from human WAT (Gregoire *et al.,* 1998; Wang *et al.,* 2005). However, as discussed above, these preadipocytes are only able to differentiate in limited passages and their differentiation ratio is largely dependent on donor age.

1.5.5.2.c Preadipocyte cell lines

Clonal cell lines have been characterized into three types according to the origin of the cells (Ailhaud, 1997). Firstly, the totipotent embryonic stem cells (ES cells) are the cell lines, which are capable of generating all cell lineages. Secondly, the generated multipotent stem cells are the cell lines, which are able to be further differentiated into myogenic, chondrogenic and adipogenic lineages. Finally, the adipoblasts are the adipogenic lineage which are the only precursors induced to adipocytes. Theoretically, clonal cells can be passaged and subcultured indefinitely (Ntambi & Young-Cheul, 2000). The clonal cells are likely to be homogeneous and can be induced at the same stage. Moreover, the clonal adipocytes are histologically and functionally similar to mature adipocytes derived from fresh adipose tissue. However, clonal cell lines are of aneuploid lineage, and this will not make them entirely the same as adipocytes derived directly from adipose tissue (Gregoire *et al.,* 1998).

3T3-Ll and 3T3-F442 are the most well-characterized murine clonal adipocyte cell lines at present. They are derived from the heterogeneous Swiss T3 cells obtained from mouse embryos (Green & Meuth, 1974). Cultured preadipocytes have an ability to constantly proliferate until reaching confluence, a stage termed as growth arrest (Rosen & Spiegelman, 2000). Differentiation of cultured preadipocytes is initiated after the postconfluent cells are exposed to a combination of agents including insulin, isobutylmethylxanthine (IBMX) (a cAMP phosphodiesterase inhibitor) and dexamethasone (a synthetic glucocorticoid agonist) (Rosen & Spiegelman, 2000).

The development of a human clonal adipocyte cell line has not been as successful as in the mouse. To date, there is still a lack of a well-characterized human preadipocyte cell line. Currently, a valuable resource is the SGBS cell strain which is developed from the SV fraction within the subcutaneous adipose tissue of an infant with Simpson-Golabi-Behmel syndrome (SGBS) (Wabitsch *et al.,* 2001). It is a rare X-linked genetic disorder with multiple abnormalities and overgrowth including adipose tissue (Wabitsch *et al.,* 2001). The fibroblast-like SGBS preadipocytes have a high capacity to be induced to adipocytes after incubation with adipogenic compounds (Wabitsch *et al.,* 2001). More importantly, the differentiated adipocytes biochemically and functionally mimic the primary adipocytes originated from adipose tissue which makes them useful as an alternative tool of primary culture

(Wabitsch *et al.,* 2001). In this thesis, the 3T3-L1 and SGBS cell strains were employed to perform most of the *in vitro* work. However, it should be pointed out that SGBS cell strain cannot be fully regarded as a cell line because it is not immortal and its differentiation capacity diminishes after 70 generations (Wabitsch *et al.,* 2001).

Differentiation of the mesenchymal stem cells into adipocytes has also been examined under *in vitro* conditions. The mesenchymal stem cells are primarily isolated from bone marrow and are able to differentiate into multiple cell types including osteoblasts, chondroblasts and adipocytes (Vaananen, 2005). Several studies suggest that bone marrow is not the only place where the mesenchymal stem cells are present because similar types of cells have been identified in many other tissues (Vaananen, 2005). Recently, a study has reported that the multipotent stem cells isolated from the SV fraction of WAT of an infant, termed hMADS cells, appear to be able to enter into an adipose lineage which is further induced to fat cells under appropriate stimuli. It is also suggested that the preadipocytes preserve their ability to be differentiated into adipocytes over 160 generations (Rodriguez *et al.,* 2004). However, due to the absence of follow-up studies, the utility of hMADS cells is still uncertain.

1.6 Endocrine function of WAT

It has only been over the last decade that the prevalent view of white adipose tissue has changed from that of it being a relatively inactive tissue, storing and releasing nutrients, as and when required, to that of an active endocrine organ with pleiotropic roles affecting a range of physiological processes. Adipose tissue is now recognised as the source of a wide range of secreted proteins and other factors (Trayhurn & Beattie, 2001; Trayhurn & Wood, 2004). The proteins released from adipocytes are referred to as adipokines; a previously used term, adipocytokines, is potentially misleading as it might be inferred that all adipocyte-secreted proteins are cytokines. A key feature of adipokines is that they are secreted proteins, and the term should therefore not be employed to describe cytosolic or membrane proteins that are not released by adipocytes. The description is also restricted to proteins secreted by adipocytes themselves, rather than adipose tissue as a whole, since this would otherwise include protein signals emanating from other cell types present within

WAT (such as macrophages) that may also be present in other tissues (Trayhurn & Wood, 2004). The secretory products of WAT are summarised in Fig. 1.2, and several will be presented in more detail below.

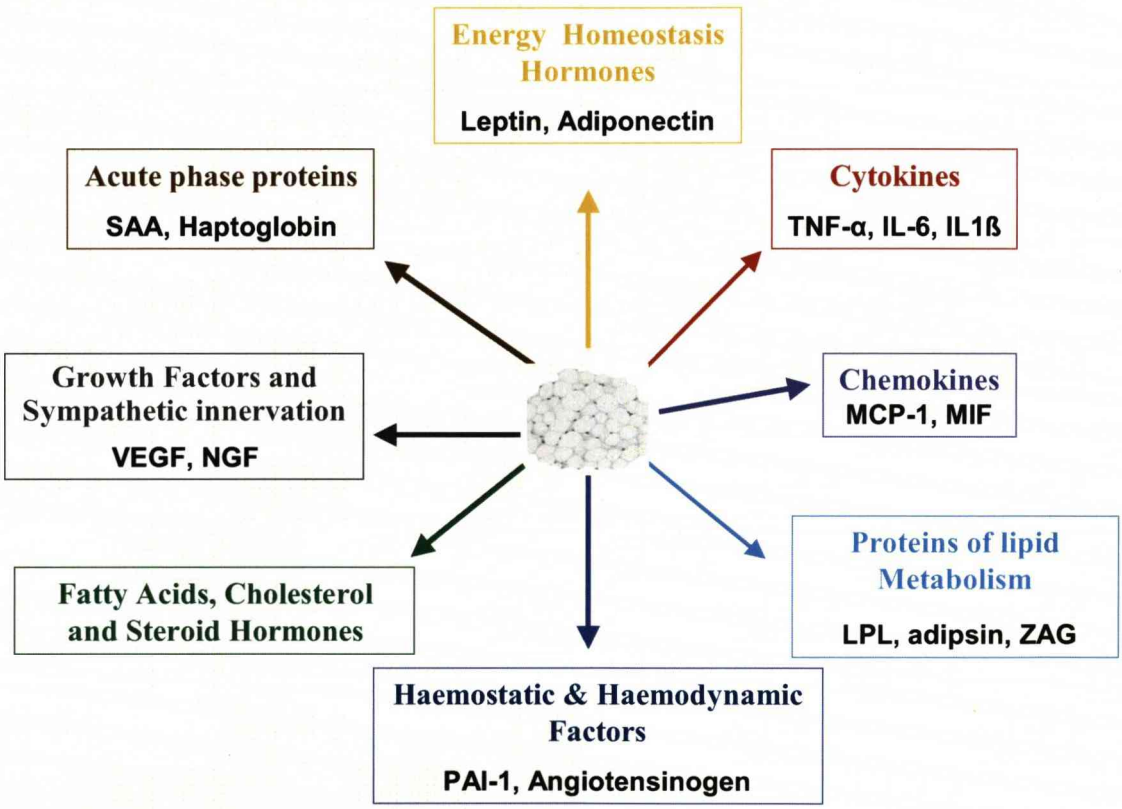
It should first be mentioned, however, that fatty acids are quantitatively the primary secreted products from WAT, being the result of TAG lipolysis mediated by HSL. Cholesterol is also released into the circulation by WAT, and although the tissue is unable to synthesise steroids *de novo,* WAT expresses the enzymes necessary for the conversion of oestrone into oestradiol, androstenedione into testosterone and androgens into oestrogens; these are therefore also secretory products of WAT (Trayhurn & Beattie, 2001).

1.6.1 Leptin

Leptin is the product of the *ob* gene, and its discovery in 1994 (Zhang *et al.*, 1994) has been pivotal in the development of the current understanding of the endocrine function of WAT (Trayhurn & Beattie, 2001). The *ob* gene encodes an 18 kDa molecule, and mature leptin is a 16 kDa cytokine-like protein (Considine & Caro, 1996). Although WAT is the primary source of leptin production, reflected by the close correlation between the degree of adiposity and basal leptin level, other tissues are known to synthesise and secrete the protein, including placenta (Hoggard *et al.,* 1997), BAT (Moinat *et al.,* 1995), stomach (Bado *et al.,* 1998) and skeletal muscle (Wang *et al.,* 1998).

Along with insulin, leptin fulfills the criteria laid out for a satiety factor as described by the lipostatic theory of energy balance referred to earlier (Kennedy, 1953). The parabiosis studies of Coleman in the 1970s also foreshadowed the discovery of leptin. Grafting of a lean control mouse to an *ob/ob* mouse led to normalisation of the latter's phenotype (Coleman, 1973; Coleman, 1978). This was interpreted as evidence that the *ob* gene encoded a humoral factor that regulated nutritional status, a mutated version of which was present in the *ob/ob* mouse. In a parallel study, Coleman demonstrated that parabiosis of a *db/db* mouse with a lean control had no effect on the former's phenotype, suggesting that the *db/db* mouse possessed a defective gene that encoded a receptor for the putative circulating satiety factor.

Fig 1.2 Products released by white adipocytes



The secretory and endocrine function of white adipose tissue. The figure shows a range of adipose tissue derived factors in energy balance, blood pressure regulation, angiogenesis, insulin sensitivity, immunity and inflammation. TNFα, tumour necrosis factor-α; IL, interlukin; PAI-1, plasminogen activator inhibitor-l; SAA, serum amyloid A; MCP-l, monocyte chemoattractant protein-l; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; MIF, macrophage migratory inhibitory factor; LPL, lipoprotein lipase; ZAG, zinc-α2-glycoprotein.

The control animal died of starvation, indicating that in response to the lack of a functional receptor, the *db/db* mouse was over expressing the satiety factor, leading to anorexia in the lean mouse (Coleman, 1973; Coleman, 1978).

Although examples of human obesity due to mutations in the *ob* gene have been documented (Montague *et al.,* 1997; Strobel *et al.,* 1998; Farooqi *et al.,* 1999), these are rare occurrences and in the vast majority of cases leptin levels are actually increased in human obesity (Considine *et al.,* 1996), leading to the concept of 'leptin resistance' in which increasing leptin levels fail to reduce food intake or increase energy expenditure. The mechanisms underlying this postulated resistance to the actions of leptin in human obesity have not been established, but two possible explanations are a failure of circulating leptin to reach its targets in the brain, and inhibition of the intracellular leptin receptor signalling cascade (Mtinzberg & Myers, 2005).

1.6.2 Proteins of lipid and lipoprotein metabolism

Lipoprotein lipase (LPL), the first protein to be identified as being secreted by adipocytes, hydrolyses circulating TAGs to release fatty acids for uptake by adipocytes. Its expression is stimulated by insulin and glucocorticoids (Trayhurn & Beattie, 2001). WAT is also an important source of cholesteryl ester transfer protein (CETP; required for accumulation of cholesteryl esters by adipose tissue), phospholipid transfer protein (PLTP; involved in cholesterol and HDL metabolism) and apolipoprotein E (ApoE; involved in cholesterol and VLDL metabolism) (Jiang & Bruce, 1995; Trayhurn & Beattie, 2001; Westerterp *et al.,* 2006).

Adipocytes secrete adipsin and acylation-stimulating protein (ASP), enabling them to regulate TAG synthesis. Adipsin is equivalent to complement D, the rate-limiting enzyme of the alternative complement pathway, and its expression is regulated by glucocorticoids and insulin (Choy *et al.,* 1992; Ahima & Flier, 2000). It is required for the synthesis of ASP, which is derived from the end product of the alternative complement pathway, complement C3a (Trayhurn & Beattie, 2001). ASP is involved in the uptake and esterification of fatty acids to TAGs for storage by adipocytes, and has an additive effect with that of insulin by allowing adipose tissue to self-regulate its lipid-storage capacity (Cianflone *et al.,* 1999). Plasma concentrations of both adipsin and ASP are increased in human obesity (Cianflone *et al.,* 1999; Ahima &

Flier, 2000).

Zinc-α2-glycoprotein (ZAG) is overexpressed in several types of malignant tumour, and has been used as a cancer marker. ZAG mRNA and protein have recently been shown to be detectable in murine and human WAT, with mRNA levels in 3T3-L1 adipocytes being increased by administration of the β3-adrenoceptor agonist BRL 37344 and the synthetic glucocorticoid dexamethasone. Furthermore, ZAG mRNA and protein levels were shown to be selectively elevated in the WAT of mice with cancer cachexia. Together with evidence that ZAG administration increases free fatty acid levels *in vivo,* and stimulates lipolysis *in vitro,* it has been suggested that ZAG may be involved in lipid metabolism and the marked loss of adiposity that occurs in the presence of certain malignancies (Bing *et al.,* 2004).

Retinol binding protein (RBP) is highly expressed by WAT, although liver and kidney are the major contributors to the circulating level of this protein, required for the transport of retinol (vitamin A) from the liver and adipose tissue to target tissues (Trayhurn & Beattie, 2001). It has recently been reported that elevated serum RBP levels in humans and mice may contribute to the development of insulin resistance in obesity and type II diabetes (Yang *et al.,* 2005). Fasting-induced adipose factor (FIAF) is an angiopoietin-like protein which was originally identified as a result of it being a target gene for PP*AR*α. Although the functions of FIAF are not yet well understood, circulating levels generally increase on fasting (Kersten *et al.,* 2000; Dutton and Trayhurn, 2007), and it has been shown to inhibit lipoprotein lipase activity, with implications for TAG accumulation by adipocytes (Backhed *et al.,* 2004). Fasting also causes increased expression of FIAF mRNA in the pituitary, suggesting a possible central regulatory role in energy balance (Wiesner *et al.,* 2004).

1.6.3 Insulin sensitivity and insulin resistance

Adiponectin (also referred to as Acrp30, GBP28, AdipoQ and apM1) is one of the most abundantly expressed adipose-specific proteins (Maeda *et al.,* 1997). Unlike most adipokines, adiponectin levels are inversely related to body weight, with reduced expression occurring in obesity and type II diabetes (Arita *et al.,* 1999). There also appear to be inverse correlations between the plasma adiponectin concentration and risk factors for cardiovascular disease, such as blood pressure (Adamczak *et al.,* 2003; Matsuzawa, 2005). Adiponectin acts as an insulin sensitiser,

stimulating glucose utilisation and fatty acid oxidation in mice (Yamauchi *et al.,* 2002). Administration of TZDs has been shown to increase adiponectin levels, and it has been proposed that adiponectin may be mediating the insulin-sensitising effects of these PPARγ agonists (Maeda *et al.,* 2001). Several anti-inflammatory and anti-atherogenic effects have been demonstrated for adiponectin, including inhibition of NFκB activation and direct effects on vascular endothelial cells to inhibit monocyte adhesion (Ouchi *et al.,* 2000; Diez & Iglesias, 2003; Matsuzawa, 2005).

Resistin (also described as FIZZ3 and ADSF) is a recently discovered adipokine that was initially proposed to act as a strong link between obesity and diabetes by inducing insulin resistance (Steppan *et al.,* 2001). This was based on observations that genetically obese and diet-induced obese mice had raised resistin levels and that immunoneutralisation of resistin in these animals had a corrective effect on their insulin resistance and hyperglycaemia. In addition, when resistin was administered to normal mice, glucose tolerance and insulin sensitivity were impaired, and treatment of 3T3-L1 adipocytes with TZDs decreased resistin gene expression (Steppan *et al.,* 2001). In humans, administration of resistin results in the development of hepatic, but not peripheral, insulin resistance (Rajala *et al.,* 2003). Other reports however, have failed to establish either clear mechanisms by which resistin expression is regulated, or a definite physiological role for the protein (Stumvoll & Haring, 2002; Yang *et al.,* 2003). A study in 2001 showed that resistin was barely detectable in human adipocytes, and no differences were found in resistin gene expression when comparing cells from normal, insulin-resistant or type II diabetic individuals (Nagaev & Smith, 2001). Other work reported that resistin levels were actually decreased in obese hyperinsulinaemic *db/db* mice compared to lean controls, and that administration of metformin (an anti-diabetic agent with insulin sensitising effects) stimulated resistin expression (Fujita *et al.,* 2002). It has been suggested that mouse and human resistin may exert divergent effects, and that the physiology of one may not be pertinent to the other, although identification of the molecular target of resistin may improve understanding of its function (Steppan & Lazar, 2004).

Visfatin (also known as PBEF) is another novel adipokine which has a proposed role in glucose homeostasis. This protein was found to be highly expressed in the visceral WAT of both humans and mice, with the circulating level increasing with adiposity (Fukuhara *et al.,* 2005). It was also determined that visfatin is a ligand for the insulin

receptor, and that it has insulin-like effects; glucose uptake was enhanced in 3T3-L1 cells, and plasma glucose concentrations were suppressed in mice, although unlike insulin, visfatin levels were unaffected by fasting or feeding (Fukuhara *et al.,* 2005).

1.6.4 Tumour necrosis factor-α

Tumour necrosis factor-α (TNFα) was first isolated in 1984 and is so named due to its ability to regress tumour masses in patients with bacterial infections of their tumours (MacEwan, *et al.,* 2002). It is a pleiotropic cytokine expressed as a 26 kDa transmembrane protein that can be cleaved to release a 17 kDa soluble form that has roles in cell apoptosis, proliferation, differentiation, inflammation and immunity (MacEwan, *et al.,* 2002). TNFα has been implicated in the pathogenesis of a range of disorders including rheumatoid arthritis, asthma, septic shock and cancer cachexia (MacEwan, *et al.,* 2002). The primary source of production is activated macrophages, but TNFα production in WAT is well documented (Hotamisligil *et al.,* 1993). However, whilst TNFα is recognised as an adipokine, being synthesised and released from adipocytes themselves, it may be that, at least in humans, the majority of the production occurs in the stromal vascular cells of adipose tissue (Fain *et al.,* 2004).

The circulating level of TNFα is increased in obesity, and it has been implicated in the development of insulin resistance, (Hotamisligil *et al.,* 1993; Hotamisligil *et al.,* 1995). This has been suggested to occur at the level of the adipocyte itself, through autocrine and paracrine actions of TNFα resulting in changes to the way adipocytes metabolise glucose and lipid. It has been shown in human adipocytes that TNFα treatment results in decreased GLUT4 mRNA and protein levels, thereby inhibiting insulin-mediated glucose uptake, and also reduces LPL mRNA levels (Hauner *et al.,* 1995). TNFα may also increase insulin resistance by elevating plasma free fatty acid concentration, not only by reducing LPL activity to decrease uptake, but also via a lipolytic effect to stimulate fatty acid release from adipocytes (Feingold *et al.,* 1992; Souza *et al.,* 2003; Ryden *et al.,* 2004).

Further evidence of the role of TNFα in inducing insulin resistance is provided by studies involving the use of TNFα-deficient mice. Mice deficient in TNFα activity due to a targeted mutation of the TNFα gene displayed significantly improved insulin sensitivity and reduced circulating free fatty acids in response to diet-induced obesity

compared to their controls (Uysal *et al.,* 1997). By eliminating TNFα signalling in *ob/ob* mice (by targeted deletions in the two TNFα receptor genes), improvements were observed in hyperglycaemia, hyperinsulinaemia and insulin sensitivity (Uysal *et al.,* 1997).

Within adipose tissue itself, TNFα is a key regulator of the expression of other adipokines. It has been shown to stimulate leptin production, leading to increased circulating leptin levels in both rodents and humans (Sarraf *et al.,* 1997; Zumbach *et al.,* 1997), and also increases synthesis of IL-6 (Wang *et al.,* 2005), PAI-l (Birgel *et al.,* 2000) and haptoglobin (Chiellini *et al.,* 2002; Oller do Nascimento *et al.,* 2004) in adipocytes. In addition, TNFα has been proposed as a key regulator of adipose tissue mass (Wame, 2003), since as well as its lipolytic actions it is recognised to induce apoptosis in mature adipocytes (Coppack, 2001), and appears to have anti-adipogenic effects via suppression of C/EBPα and PPARγ expression (Sethi & Hotamisligil, 1999).

1.6.5 Interleukins

Adipose tissue is recognised as the source of a range of interleukins, which are key regulators of the inflammatory response and modulate the expression of a range of other adipokines. IL-6 has a ubiquitous tissue distribution, and is a major cytokine with effects on cell growth, acute phase responses and carbohydrate and lipid metabolism (Mohamed-Ali *et al.,* 2001). It is expressed and secreted by adipocytes, and although it has local effects, it is released into the circulation and may also act centrally in the hypothalamus to regulate energy balance (Trayhurn & Wood, 2004). Circulating IL-6 levels are elevated in obesity and insulin resistance, and sympathetic stimulation has been shown to increase plasma IL-6 concentration in both humans and mice, with administration of sympathetic agonists elevating production of the interleukin by human and murine adipocytes (Mohamed-Ali *et al.,* 2001). Adipose expression of IL-6 is stimulated by insulin (Krogh-Madsen *et al.,* 2004) and TNFα (Wang *et al.,* 2005), and the cytokine induces insulin resistance in adipocytes, which can be prevented by the TZD rosiglitazone (Lagathu *et al.,* 2003; Rotter *et al.,* 2003). Other interleukins synthesised within WAT include IL-1β, IL-8, IL-10 and IL-18 (Trayhurn & Wood, 2004). IL-1β has been shown to mediate leptin induction in mice administered inflammatory stimuli (Faggioni *et al.,* 1998), and also stimulates IL-6

release from human adipocytes (Flower *et al.,* 2003). IL-8 may be involved in atherogenesis, and circulating levels are correlated with obesity; its release from adipose tissue is stimulated by IL-1β and TNFα, but inhibited by dexamethasone (Bruun *et al.,* 2001; Trayhurn & Wood, 2004). The anti-inflammatory cytokine IL-10 is secreted by human WAT explants, where it is upregulated by LPS and TNFα, and its expression in WAT is raised in both human and rodent obesity (Juge-Aubry *et al.,* 2005). The plasma concentration of IL-18, a pro-inflammatory cytokine, is also elevated in obesity, and has been shown to fall in response to weight reduction (Esposito *et al.,* 2002). It has recently been established that WAT is indeed a site of IL-18 expression, with mRNA levels in cultured human adipocytes markedly stimulated by treatment with TNFα, although no evidence was found for the release of IL-18 from these cells (Wood *et al.,* 2005). Another study has, however suggested that IL-18 is released from human adipocytes (Skurk *et al.,* 2005)

1.6.6 Haemostatic and haemodynamic factors

The pro-thromboembolic factor plasminogen activator inhibitor-l (PAI-1) is a key regulator of vascular haemostasis; by inhibiting plasminogen activation, it suppresses the formation of plasmin which is involved in fibrinolysis (Mutch *et al.,* 2001). Plasma PAI-l concentrations are elevated in obesity, and production of the factor in WAT has been demonstrated in both rodents and humans. The increased production of PAI-l in obesity has been linked to higher levels in WAT of several factors known to stimulate PAI-l expression, including TNFα, TGFβ and IL-6, and PAI-l has been implicated in the increased risk of cardiovascular and atherosclerotic complications associated with obesity (Mutch *et al.,* 2001; Trayhurn & Wood, 2004).

Tissue factor (TF) is the main cellular initiator of the coagulation cascade and also acts as a cell-surface receptor for the activation of factor VII. Abnormal expression of TF may promote thrombotic events through activation of the coagulation cascade. TF gene expression is raised in the adipose tissues of *ob/ob* and *db/db* mice compared with their lean controls, and TF mRNA levels are increased in adipocytes by administration of TGFβ and insulin. It has therefore been proposed that the hyperinsulinaemia associated with obesity may induce TF expression in WAT, and that elevated TF levels may contribute to the prothrombotic state existing in the obese (Samad *et al.,* 1998; Samad *et al.,* 2001).

The renin-angiotensin system (RAS) is an important regulator of systemic blood pressure and renal electrolyte homeostasis, with local RAS activity implicated in pathological changes of organ structure and function by modulation of gene expression and inflammatory responses (Engeli *et al.,* 2000). Angiotensinogen (AGT) is converted by renin to angiotensin II (Ang II), which is a potent vasoconstrictor and key regulator of vascular tone. Although the liver is the main source of AGT, it is produced and secreted by adipose tissue, with adipocyte expression and secretion of AGT stimulated by glucocorticoids but inhibited by insulin and TNFα (Aubert *et al.,* 1997; Aubert *et al.,* 1998; Wang *et al.,* 2005). AGT levels have been shown to be elevated in rodent models of obesity, and weight reduction in humans resulted in reduced circulating AGT, as well as a fall in AGT expression in adipose tissue (Engeli *et al.,* 2005). The increased plasma AGT concentration seen in obesity may thus be a significant contributor to the hypertension associated with this condition (Engeli *et al.,* 2000; Engeli *et al.,* 2005).

1.6.7 Acute phase proteins

The main source of acute phase proteins is the liver, but several are also synthesised and secreted from adipose tissue. Haptoglobin is expressed in both human and mouse white adipocytes, with expression being stimulated by TNFα and IL-6, and inhibited by TZDs (Chiellini *et al.,* 2002; Oller do Nascimento *et al.,* 2004). Circulating levels of haptoglobin are elevated in obesity, which may contribute to the state of chronic mild inflammation (Chiellini *et al.,* 2002). It is capable of binding haemoglobin, thus preventing iron loss and renal damage, and also acts as an antioxidant and angiogenic factor, which may be its main roles in adipose tissue (Oller do Nascimento *et al.,* 2004). Antioxidant and angiogenic functions have also been proposed for metallothionein, a metal-binding stress-respone protein which is expressed and secreted in WAT (Trayhurn *et al.,* 2000), and whose expression is stimulated by TNFα in human white adipocytes (Wang *et al.,* 2005).

WAT has recently been identified as a significant source of serum amyloid A (SAA), an acute phase protein recognised as an independent risk factor for coronary artery disease, as well as contributing to the development of amyloidosis. Serum SAA levels have been reported to be correlated with BMI, adiposity and adipose tissue

SAA mRNA levels (Sjoholm *et al.,* 2005). WAT expression of the gene, and circulating levels of the protein, were also shown to be under nutritional regulation, with decreases in both occurring in response to weight loss, thus indicating that SAA could be a link between obesity and cardiovascular disease (Poitou *et al.,* 2005; Sjoholm *et al.*, 2005). Another acute phase protein whose circulating level is correlated with BMI is C-reactive protein (CRP) (Bulló *et al.,* 2003), recognised as a marker of inflammation and atherosclerosis (Lind, 2003). It has been thought that the elevated serum levels of CRP present in obesity are in part due to increased secretion from WAT. Moreover it has recently been suggested that human adipocytes secrete CRP and inflammation may modulate its gene expression (Memoli *et al.,* 2007).

1.6.8 Chemokines

It has been reported that obesity is associated with an increased level of macrophages infiltrating WAT, leading to the increased expression of many inflammation and macrophage-specific genes within the tissue (Weisberg *et al.,* 2003; Xu *et al.,* 2003). This infiltration process may be regulated by chemokines secreted from adipocytes, one of which is macrophage migratory inhibitory factor (MIF), an important mediator of inflammatory responses. Human adipocytes express and secrete MIF, and as the level of production is correlated to BMI it has been proposed that MIF may be an obesity-dependent regulator of macrophage infiltration into adipose tissue (Skurk *et al.,* 2005; Trayhurn, 2005).

Another factor which may play a key role in the infiltration of WAT by macrophages is monocyte chemoattractant protein-l (MCP-l). *In vivo* studies have demonstrated that MCP-l is overexpressed in obese *(ob/ob)* mice compared with their lean controls, and that WAT is a major source of MCP-l (Sartipy & Loskutoff, 2003). Recent work has shown MCP-l mRNA levels and protein secretion to be stimulated by TNFα in human adipocytes (Wang *et al.,* 2005). Insulin stimulates MCP-l release from 3T3-Ll adipocytes, and also increases circulating levels of MCP-l in *ob/ob* mice, which may link the chemokine with the pathologies seen in obesity and hyperinsulinaemia (Sartipy & Loskutoff, 2003).

1.7 PHYSIOLOGICAL ROLE OF GASTROINTESTINAL HORMONES IN THE CONTROL OF ADIPOSE TISSUE METABOLISM

Little is known of any physiological role of gastrointestinal hormones in the control of lipid deposition and mobilisation in adipose tissue. However, a limited number of investigations have demonstrated that these hormones may have an important role in the control of pathways of lipid metabolism. The control may be consequent to changes in the secretion of insulin or directly by controlling lipogenesis, lipolysis or the activity of lipoprotein lipase. Most of this knowledge comes from *in vitro* studies and the problems associated with tissue viability and pharmacological doses of hormones need to be considered in the interpretation of data obtained.

1.7.1 White adipose tissue Metabolism

1.7.1.1 Glucose metabolism

Glucose, the substantial energy source within the body, fluctuates in a narrow range in the bloodstream during fasting and feeding. Dysregulation of the balance between glucose intake and output leads to a high glucose level in the circulation. Muscle and WAT are the crucial peripheral organs to reduce the increased circulating glucose level after a meal by uptake of glucose into the cells. In fact, glucose is unable to be independently transported into the cells. It must be supported by a group of facilitative glucose transporters (GLUTs) - to cross the cell membrane. To date, at least 14 GLUT family members have been identified, and many of them, such as GLUT 1, GLUT3-5, GLUTl0, GLUT12, and HMIT, are present in white adipocytes (Wood & Trayhurn, 2003; Trayhurn *et al.,* 2006). GLUT1, which is found ubiquitously, is located on the plasma membrane of adipocytes and probably mediates basal, non-insulin mediated glucose uptake (Khan & Pessin, 2002). GLUT4, the most important insulin-responsive glucose transporter, is expressed in skeletal muscle, heart muscle and adipocytes (Khan & Pessin, 2002). Under basal conditions, GLUT4 is stored as intracellular vesicles; however, the activation of the insulin signalling pathway triggers the translocation of GLUT4 from its storage vesicles in the cytosol to the plasma membrane, and hence facilitates increased

glucose uptake into the cells (James *et al.,* 1994).

Normal function of the insulin signal transduction pathway in target cells is essential to maintain the circulating glucose at a stable level. However, in type II diabetes, impaired insulin sensitivity results in an enhanced glucose concentration in the bloodstream. The precise mechanism of insulin resistance remains to be fully understood. However, obesity has been indicated to be a major risk factor for type II diabetes.

1.7.1.2 Lipid metabolism

Triglycerides, also known as triacylglycerols (TAGs), are the core energy sources stored in WAT originally derived from foods (Trayhurn & Beattie, 2001). The formation of chylomicrons after a meal facilitates the transportation of TAGs from the small intestine to adipose tissue for storage. Also, the liver synthesizes TAGs which in turn are exported and stored in adipose tissue. In addition, TAGs in adipocytes can be derived and synthesized from glucose taken up by adipocytes.

If energy intake is insufficient for requirements, TAGs are broken down to supply the extra energy by a process known as lipolysis (Arner, *et al.,* 2005). TAGs are hydrolysed by activation of the hormone sensitive lipase (HSL) to release free fatty acids (FFAs) and glycerol (Fig 1.3). Both FFAs and glycerol can cross the plasma membrane of adipocytes and be secreted into the circulation. FFAs loosely combine with serum albumin in the bloodstream. Glycerol is unable to be taken up by adipocytes after it is released; thus, to measure the amount of glycerol in the circulation or the culture medium of the cells is a useful method to estimate the lipolytic activity of the cells.

HSL is a rate-limiting enzyme activating lipolysis through the cyclic adenosine monophosphate (cAMP) dependent protein kinase pathway (Arner, *et al.,* 2005).

Evidence strongly suggests that catecholamines, the neurotransmitters released by the nerve endings of the SNS, may be the key mediators of lipolysis (Arner, *et al.,* 2005). The binding of catecholamines to β-adrenoceptors on the cell membrane activates the adenylate cyclase, an enzyme having an ability to convert adenosine triphosphate (ATP) to cAMP (Carmen & Victor, 2006). The increased cAMP further stimulates activation of the cAMP-dependent kinase (PKA), which further phosphorylates its downstream molecules, such as perilipin A (Peri A), the most

prevalent substrate of PKA (Carmen & Victor, 2006). The phosphorylation of perilipin is an essential process for translocation of the HSL from the cytosol to lipid droplets, and finally the breakdown of lipid (Souza *et al.,* 2002; Miyoshi *et al.,* 2006).

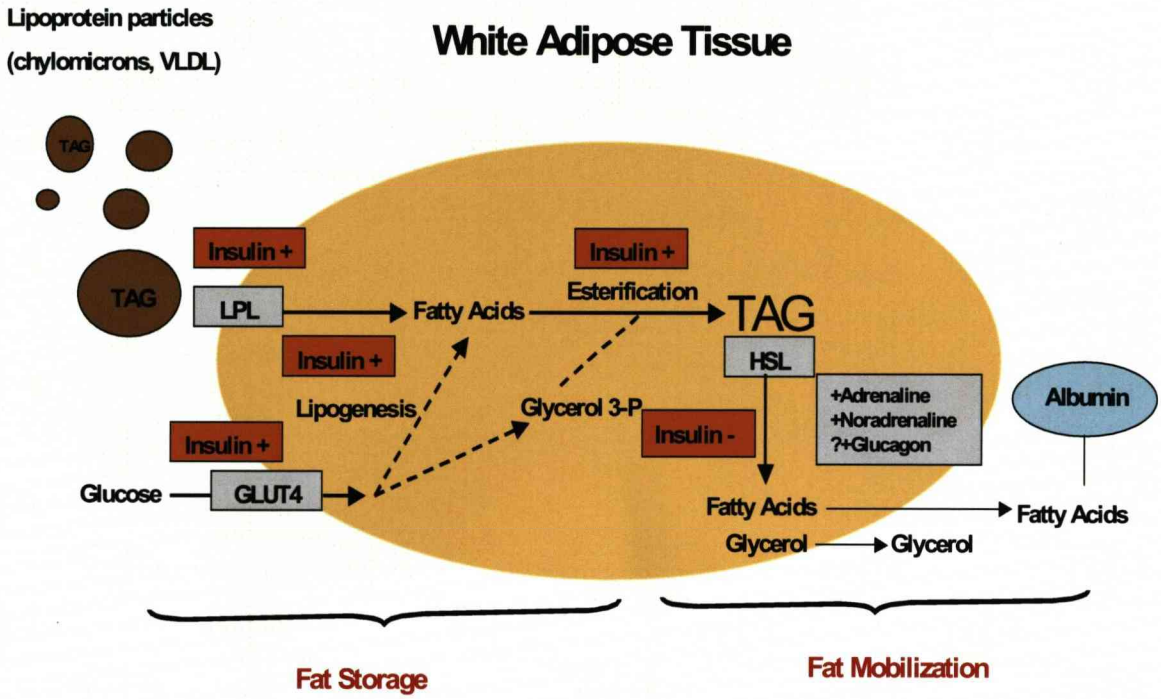
It is believed that there are metabolic and functional differences between subcutaneous and visceral fat depots (Arner *et al.,* 2005). Catecholamine-induced lipolysis is higher in visceral fat than in subcutaneous fat, but the basal lipolytic activity is opposite (Arner *et al.,* 2005). It is thought that a high rate of hormone-stimulated lipolysis in visceral fat leads to a large amount of free fatty acid release reaching the liver and other metabolic organs, such as the heart and skeletal muscle, which is the major cause of the impairment of glucose tolerance in obesity (Klein *et al.,* 2004).

As discussed above, lipolysis is mainly regulated by the neurotransmitter nonadrenaline released from the SNS. In addition to the SNS, lipolysis is also influenced by several other factors, such as the reduced plasma glucose concentration during fasting, increased growth hormone and glucocorticoids, which all stimulate free fatty acid release by adipocytes whereas lipolysis is inhibited by the elevated insulin (Ahima & Flier, 2000).

HSL has long been regarded as the only lipase mediating lipid hydrolysis in cells. However, recent studies have shown that the hormone-mediated-lipolytic activity still exists at cellular levels in HSL-knockout mice (Osuga *et al.,* 2000). This finding indicates that there may be other unidentified intracellular triacyglycerol lipase(s).

This hypothesis has been confirmed following the discovery of a novel adipose-expressed triacyglycerol lipase (ATGL) (Zimmermann *et al.,* 2004). ATGL, also known as patatin-like phospholipase domain containing protein 2 (PNPLA 2) or desnutrin, is highly expressed in adipose tissue and has a specific role to break down lipid by removing fatty acids from TAGs (Zimmermann *et al.,* 2004). Subsequent studies suggest that ATGL is downregulated in white adipocytes in obesity and likely to mediate the basal lipolysis activity (Langin *et al.,* 2005). Knockout of the ATGL gene in mice leads to enlarged fat depots, including gonadal, inguinal WAT and BAT; moreover, TAGs have been found to accumulate in cardiac muscle, testes and kidney (Haemmerle *et al.,* 2006), indicating that ATGL has an essential role in the regulation of lipolysis.

Fig 1.3 Fatty acid and glucose metabolism in WAT



Adapted from Frayn,et al.,1996

A schematic pathway of how the dietary fat digested and then re-synthesised into TAG is carried in the circulation as lipoproteins. Lipoprotein molecules are transported in to bloodstream. Fat then enters adipose tissue and is hydrolysed by lipoprotein lipase, the activity of which is stimulated by insulin. When required, the stored TAG is mobilised by HSL, which is stimulated by adrenaline and glucagon. When the fatty acids are released from adipose tissue, they are transported and attached to the major protein in the circulation, which is albumin. HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; TAG, triacylglycerol

1.7.2 Functions of gastrointestinal hormones in WAT metabolism

1.7.2.1 Indirect control via regulation of insulin secretion

Insulin is known to stimulate lipogenesis (Stansbie *et al.,* 1976), the activity of lipoprotein lipase (Cryer *et al.,* 1976; Sadur and Eckel, 1982; Eckel, 1987) and to have an antilipolytic effect (Denton *et al.,* 1981). Therefore, any hormones that affect the secretion of insulin will indirectly affect adipose tissue metabolism via these mechanisms. Several gastrointestinal hormones are known to stimulate the release of insulin and their role in the enteroinsular axis is well established. The most important hormones fulfilling this role are GIP (Dupré *et al.,* 1973; Anderson *et al.,* 1979; Elahi *et al.,* 1979) and GLP-1 (7-36) amide (Kreymann *et al.,* 1987). GIP increases insulin binding and insulin-dependent glucose uptake (Starich *et al.,* 1985; Hauner *et al.,* 1988). GLP-1 potently increases insulin-dependent glucose uptake in adipocytes at low concentrations (Egan *et al.*, 1994; Miki *et al.*, 1996; Perea *et al.*, 1997). It also rescues the downregulation of glucose transporter-4 (GLUT-4) brought about by prolonged insulin exposure (Wang *et al.*, 1997).

Other hormones that have been demonstrated to stimulate insulin release include cholecystokinin (Unger *et al.,* 1967; Dupré *et al.,* 1969). Neurotensin (Brown and Vale, 1976) and somatostatin (Pederson, Dryburgh and Brown, 1975) have also been shown to inhibit the secretion of insulin.

In 3T3-L1 adipocytes, ghrelin enhances insulin sensitivity (Kim *et al.*, 2004) as measured by glucose transport. However in a diverse study, using brown adipocytes, insulin-induced glucose uptake was not altered by ghrelin treatment (Ott *et al.*, 2002).

1.7.2.2 Control of lipogenesis

Recently, it has become apparent that GIP may have direct effects on metabolism, in particular anabolic effects, and not solely indirectly via its stimulation of insulin release (Beck, 1989).

GIP has been found to exert a direct influence on the insulin sensitivity of rat adipocytes. The incubation of isolated adipocytes with GIP led to increased insulin receptor affinity. An increase in the sensitivity of insulin-stimulated glucose transport was also demonstrated (Starich, *et al.,* 1985) which is the first step in the synthesis of

triglycerides from this precursor.

It has been reported that GIP is capable of stimulating the transport of glucose into isolated rat adipocytes and the incorporation of glucose into extractable lipids (Hauner *et al.,* 1988*).*

Studies showed that GIP increased basal lipogenesis in rat (Oben *et al.*, 1991a) and lamb adipose explants (Baba *et al.*, 2000). Consistent with these findings, GIP has been noted to increase insulin induced lipogenesis in primary cultures of rat adipocytes (Beck and Max, 1983; Beck and Max, 1988) and to boost the activity of lipoprotein lipase in cultured murine preadipocytes (Eckel *et al.*, 1979). Moreover, GIP suppresses β-adrenergic and glucagon-mediated lipolysis in isolated rat adipocytes (Dupré *et al.*, 1976; Hauner *et al.*, 1988). However, GIP alone has also been reported to increase basal lipolysis in primary rat adipocytes (Beck and Max, 1983; Hauner *et al.*, 1988). Furthermore, an inhibition of insulin-induced lipogenesis has been described in lamb adipose explants (Baba *et al.*, 2000).

Another study has shown GIP to stimulate the incorporation of glucose into saponifiable fatty acids by explants of rat adipose tissue (McCarthy, 1993) and a weak lipogenic effect of GIP has also been demonstrated in ovine adipose tissue using acetate as a precursor (Haji Baba and Buttery, 1991; McCarthy, 1993).

An investigation into the effects of GLP-1 on human adipose tissue concluded that it exerts differential, concentration-dependent effects on lipid metabolism in human adipocytes. This was attributed to the existence of different GLP-1-binding receptors. At low concentrations, GLP-1 was found to act synergistically with insulin to increase lipogenesis in human adipocytes, whereas, at higher concentrations, it augmented glucagon-dependent lipolysis (Villanueva-Penacarrillo *et al.*, 2001).

Recently, Ghrelin has been found to activate the mitogen-activated protein kinase pathway *in vitro* (Kim *et al.*, 2004; Zhang *et al.*, 2004), which stimulates cellular proliferation and differentiation in cultured white adipocytes (Choi *et al.*, 2003; Kim *et al.*, 2004; Zhang *et al.*, 2004). This has been confirmed in rats *in vivo* (Thompson *et al.*, 2004). In brown adipocytes, however, ghrelin does not seem to affect adipocyte differentiation (Ott *et al.*, 2002).

1.7.2.3 Control of lipolysis

The stimulatory effect of glucagon on lipolysis has long been known after first being demonstrated in rat adipose tissue (Hagen, 1961; Vaughan, 1961). Secretin, which is related to glucagon in structure, has also been shown to stimulate lipolysis in isolated rat adipocytes (Rudman and Del Rio, 1969). This action was confirmed in a similar study where it was shown that secretin also causes an increase in cyclic AMP levels in the adipocytes (Butcher and Carlson, 1970).

The effect of GIP on lipolysis was investigated in adipocytes isolated from rat adipose tissue and was found not to stimulate lipolysis itself but was capable of inhibiting the lipolytic action of glucagon. GIP was without effect on the lipolysis stimulated by secretin. The antilipolytic effect appeared to result from competition for the glucagon receptor since GIP was able to bind non-functionally to the glucagon receptor (Dupré *et al.,* 1976).

In a more recent study, GIP has been found to have a weak lipolytic effect in adipocytes isolated from rat adipose tissue (Hauner *et al.,* 1988). This discrepancy with the previous study may arise from the different methods used to estimate lipolysis. By measuring the release of free fatty acids, GIP was shown not to stimulate lipolysis (Dupré *et al.,* 1976) whereas by measuring glycerol release it was found to be weakly lipolytic (Hauner *et al.,* 1988). It is possible that the free fatty acids released during lipolysis may become re-esterified in the adipocyte and therefore mask a weak lipolytic effect.

It has been reported that GLP-1 (7-36) amide and, to a lesser extent, GLP-1 (1-36) amide stimulated lipolysis in isolated rat adipocytes although the effect was very weak compared to that of glucagon (Ruiz-Grande *et al.,* 1992). However, one study did not find any effect of GLP-1 on lipolysis in human subcutaneous adipocytes (Bertin *et al.*, 2001). Likewise, reports on the lipolytic or lipogenic activity of GLP-1 in rodent adipocytes have been contradictory, some supporting the notion of increased lipogenesis (Oben *et al.*, 1991; Egan *et al.*, 1994; Perea *et al.*, 1997), and some the opposite (Ruiz-Grande *et al.*, 1992).

A recent study on the role of ghrelin and GHS-R on rat adipogenesis showed that ghrelin suppresses isoproterenol-induced lipolysis (Choi *et al.*, 2003).

1.7.2.4 Control of lipoprotein lipase activity

In addition to its lipogenic effect, GIP has also been shown to stimulate the release of lipoprotein lipase activity from cultured 3T3-Ll preadipocytes into the medium and also the total amount of lipoprotein lipase activity within these cells in a dose-dependent manner (Eckel, *et al.,* 1979). This was followed by demonstration that the infusion of GIP in dogs promoted the clearance of chylomicron triglycerides which had been administered in the form of chyle collected from donor dogs following a triglyceride rich meal (Wasada *et al.,* 1981). This is presumed to occur via the stimulation of lipoprotein lipase activity by GIP, lipoprotein lipase being involved in the transfer of circulating lipoprotein triglycerides to adipose tissue. It is also possible that this effect results from increased transport of free fatty acids into the adipocytes. GIP has been shown to promote the insulin-stimulated incorporation of fatty acids into rat adipose tissue; this effect occurs using physiological doses of GIP and is dose-dependent. It was suggested that GIP might exert its effect by facilitating the passage of fatty acids through the membrane of the adipocyte (Beck and Max, 1983). It has been demonstrated that GIP stimulates total cellular lipoprotein lipase activity in explants of rat adipose tissue whilst GLP-l (7-36) amide was found to be without effect (Oben *et al.,* 1991).

1.7.2.5 Endocrine activity (Secretion of adipokines)

Little is known in regards with the regulation of adipokine production by incretins. However, a recent study has now examined the possibility that specific actions of GIP are mediated through the direct regulation of resistin release in 3T3-L1 adipocytes, resulting in increasing the activity of both PKB and LPL pathways (Kim *et al.,* 2007). On the other hand, ghrelin directly suppresses adiponectin mRNA expression (Ott *et al.*, 2002). In one study, intra-peritoneal injection of ghrelin increased both adiponectin and leptin expression in adipose tissue, but these effects did not reach statistical significance (Asakawa *et al.*, 2003). Any effect of obestatin in the regulation of adipokines has not been explored to date.

1.8 AIMS AND SCOPE OF THE RESEARCH IN THIS THESIS

The survey of the literature indicates that gastrointestinal hormones released during the digestion and absorption of nutrients directly affect the partitioning of nutrients within the body. In particular gastrointestinal hormones appear to have regulatory roles in the complex lipogenic and lipolytic pathways that occur in white adipose tissue. Excessive fat deposition in the form of obesity, is now a major health problem worldwide leading to an increased risk of many diseases, including type II diabetes, heart failure and cancer. Adipose tissue was originally thought to be an organ solely for energy storage. In recent years, ongoing research has shown that lipid storage is not the only function of white fat. The discovery of leptin, which was followed by the identification of other protein factors, termed 'adipokines' secreted by white adipose tissue (WAT), indicated that WAT is major endocrine organ.

Elucidation of the roles of gastrointestinal hormones in adipose tissue function may lead to further understanding of the mechanisms of obesity which in turn could allow more effective approaches to the prevention and treatment of obesity. Thus, if specific receptors for gut peptides can be found in adipose tissue one may infer that it is likely that they may have a direct effect on adipose tissue function. While specific receptors for GIP have been identified, studies have been inconclusive with regard to GLP-1 and very little is known regarding ghrelin and obestatin.

The first objective of the work in this thesis was to establish whether the genes encoding the receptors for ghrelin (GHS-R), obestatin (GPR-39), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) are indeed expressed in adipocytes. The work then hoped to elucidate the nature of the interaction of these hormones with adipose tissue in the form of the alterations in the endocrine secretion of this organ. It was specifically hypothesised that the incretins may regulate the expression and release of key adipokines and thereby play a significant role in adipose tissue as part of the cross-talk between the gut and adipose tissue.

More specifically the aims of this research project were:

- To employ RT-PCR was used to examine whether the receptors genes are expressed in different mouse tissues, and more particularly to screen for gene

expression of these receptors in major fat depots of both mice and humans.

- To compare receptor gene expression between lean and obese subjects to assess whether this is altered in white adipose tissue in human obesity.

- To assess the gene expression for these receptors in adipocyte cell culture systems of both mouse (3T3-L1) and human (Simpson-Golabi-Behmel syndrome; SGBS), in order to establish whether these systems can be used to assess the affect of the gut hormones on adipocytes.

- To employ Western blotting to determine whether receptor protein is expressed by adipose tissue and adipocyte cell culture system of humans.

- To explore the effects of the gut peptides on the gene expression of key adipokines including leptin, adiponectin, IL-6 and MCP-1 genes in human SGBS adipocytes.

- Finally, to investigate the effects of a 24-h treatment of the gut peptides in the presence or absence of insulin, on glucose uptake using 2-deoxy-D-glucose (2-DG). This was to allow us to ascertain if prolonged exposure to these peptides had any modulatory effects by themselves or on insulin action in cultured fat cells.

CHAPTER 2
MATERIALS & METHODS

2.1 Reagents and Methods

2.1.1 Chemical reagents

Acetic acid	Sigma
Acrylamide	Sigma
α-linoleic acid	Sigma
Agarose	Sigma
Ammonium persulphate	Sigma
Arachidonic acid	Sigma
β-mercaptoethanol	Sigma
Bicinchoninic acid (BCA)	Sigma
Biotin	Sigma
Bovine serum albumin	Sigma
BRL-37344	Tocris
Bromophenol blue (BPB)	Sigma
Chloroform	Fisher
Conjugated linoleic acid	Sigma
Copper (II) sulphate solution	Sigma
Cortisol	Sigma
Dexamethasone	Sigma
Disodium hydrogen phosphate	Sigma
Docosahexaenoic acid	Fluka
Dulbecco's modified Eagle's medium/Ham's F12 (1:1)	Invitrogen
Eicosapentaenoic acid	Sigma
100% Ethanol	Sigma
Ethidium bromide	Sigma
Foetal calf serum	Biosera
Fluorescein anti-mouse IgG	Vector
Free glycerol reagent	Sigma
GeneRuler ^{TM} 100 bp DNA ladder	Helena BioSciences
Glucose	Sigma
Glycerol	Sigma
Glycerol standard solution	Sigma
Glycine	Sigma
Guanidine hydrocholoride	Sigma
HEPES	Sigma
Human recombinant Ghrelin protein	ALEXIS
Human recombinant GIP protein	PolyPeptide Laboratories
Human recombinant GLP-1 protein	PolyPeptide Laboratories
Human recombinant Obestatin protein	ALEXIS, PeptaNova, Peptides I
Human recombinant Des 1-10 Obestatin protein	Peptides International

Human polyclonal antibody to human GHS-R	Novus Biologicals
Human polyclonal l antibody to human GIP R	MBL International
Human polyclonal antibody to human GLP-1R	MBL International
Human polyclonal antibody to human GPR 39	Novus Biologicals
Human transferrin	Sigma
I-methyl-3-isobutylxanthine (IBMX)	Sigma
Insulin	Sigma
Isoprenaline Isopropanol	Sigma
Linoleic acid	Sigma
Lipopolysaccharide	Sigma
Magnesium sulphate	Sigma
Methanol	VWR
Myristic acid	Sigma
Noradrenaline	Sigma
Normal horse serum	Vector
Oleic acid	Sigma
Palmitic acid	Sigma
Pantothenate	Sigma
Penicillin/Streptomycin	Invitrogen
Photographic developer	Sigma
Photographic fixer	Sigma
Potassium chloride	Sigma
Potassium dihydrogen orthophosphate Ponceau	Sigma
Ponceau S	Sigma
Rainbow molecular weight maker	Amersham
RNase AWAY	Molecular BioProd
Rosiglitazone	GlaxoSmithKline
Saponin	Sigma
Sheep anti-rabbit conjugated to horseradish peroxidase	Sigma
Sodium chloride	Sigma
Sodium citrate	Sigma
Sodium dodecyl sulphate	Sigma
Sodium dihydrogen orthophosphate	Sigma
Sodium EDT A	Sigma
Sodium hydroxide	Sigma
Stearic acid	Sigma
Sucrose	Sigma
10xTBE buffer	Fisher
Trichloroacetic acid	Sigma
Triiodothyronine (T_3)	Sigma
Tris-Base	Sigma
Tris-hydrochloride	Sigma

Trizol	Invitrogen
Trypsin/EDT A	Invitrogen
Tween 20 (polyoxyethylene sorbitan monolaurate)	Dakocytomation
Ultra-pure water	Sigma
Vectashield	Vector

2.1.2 Kits

DNA-free Kit	Ambion
ECL Westem Blotting detection reagents	Amersham
QPCR Core Kit	Eurogentec
ReddyMix ™ Master PCR Master Mix Kit	Abgene
Reverse-iT ™ 1st Strand synthesis Kit	Abgene

2.1.3 Equipment

2011 Macrovue UV transilluminator	Ultra-Violet Products
ABI Prism 7700 real-time PCR machine	Applied Biosystems
Beckman Preparative Ultracentrifuge	Beckman
Benchmark™ Plus microplate spectrophotometer	Bio-Rad
Biophotometer	Eppendorf
Centrifuge 5415 D	Eppendorf
Centrifuge 5417 R	Eppendorf
Coming filter system (0.22 µm membrane)	Fisher
Glass homegenizers (borosilicate)	Fisher
HB-1000 hybridisation oven	Ultra-Violet Products
Horizontal Gel Electrophoresis units	SCIE-PLAS
Hybond ™ ECL	Amersham
Hyperfilm ECL	Amersham
Kodak Digital Science DC 120 digital camera	Kodak
Mx3005P™ QPCR System	Stratagene
Nitrocellulose membrane	Amersham
PCR Express thermal cycler	Hybraid
PhotoEnhancer	Kodak
3310 pH meter	Barloworld
Polytron Ultra- Turrax T25 electric homogenizer	Janke& Kunkel
Reichert Polyvar Met	Reichert
Real-time PCR plates	Abgene
Rocker	Bibby Sterilin
TV 400 vertical gel electrophoresis units	SCIE-PLAS
UVette® plastic disposable cuvettes	Eppendorf

Water bath	Grant Instruments
6-well plate	Fisher
12-well plate	Fisher
96-well microplate	Fisher

2.1.4 Software

Beacon Designer	Premier Biosoft
GraphPad InStat version 3.00 for Windows 95	GraphPad Software
Kodak Digital Science ID 1 image analysis software	Kodak
MXPro™ QPCR Software	Stratagene
Primer Express® Software	Applied Biosystem
Primer Premier 5 software	Premier Biosoft
SDS version 1.7a software	Applied Biosystems

2.1.5 Address and URLs

Addresses:

Abgene UK,ABgene House, Blenheim Road, Epsom KTI9 9AP, UK.

ALEXIS Biochemicals Coporation, AXXORA (UK) LTD.PO Box 6757, Bingham, Nottingham, NG13 8LS.

Ambion Ltd, Spitfire Close, Ermine Business Park, Huntingdon, Cambridgeshire, PE29 6XY, UK.

Amersham PLC, Amersham Place, Chalfont, Buckinghamshire, HP7 9NA, UK.

Applied Biosystems, Kelvin Close, Birchwood Science Park North, Warrington, Cheshire, WA3 7PB, UK.

Bechman Instruments Inc, Palo Alto, California 94304, USA.

Barloworld Scientific Ltd, T/As Jenway, Gransmore Green, Felsted, Dunmow, Essex, CM6 3LB, UK.

Bibby Sterilin Ltd, Stone, Staffordshire, ST15 OSA UK.

Biosera, I Acorn House, the Broyle, Ringmer, East Sussex, BN8 5NN, UK.

BioVendor Laboratory Medicine, Inc, CTPark Modrlce, Evropska 873, 664 42 Modrlce, Czech Republic.

MBL International, Inc, 15B Constitution Way, Woburn, MA 01801, USA

Dakocytomation, Dako Denmark *NS,* Produktionsvej 42, DK-2600 Glostrup, Denmark.

Eppendorf UK Ltd, Endurance House, Chivers Way, Histon, Cambridge, CB4 9ZR, UK.

Eurogentec Ltd, P.C.House, 2 South Street, Hythe, Southampton, Hampshire S045 6EB, UK.

Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LEII 5RG, UK.

Fluka, Industriestrasse 25, CH-9471 Buchs SG, Switzerland.

GlaxoSmithKline, Glaxo Wellcome UK Ltd., Stockley Park West, Uxbridge, Middlese, UB 11 I BT, UK.

Grant Instruments (Cambridge) Ltd, Shepreth, Royston, Hertfordshire, SG8 6GB, UK.

Helena BioSciences Europe, Colima Avenue, Sunderland Enterprise Park, Sunderland, Tyne & Wear, SR5 3XB, UK.

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, P A4 9RF, UK.

Janke & Kunkel, Janke-&-Kunkel-Str. 10, 79219 Staufen, Germany.

GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA.

Kodak Ltd, P.O.Box 66, Station Road, Hemel Hempstead, Hertfordshire, HPI lW, UK.

Microsoft Corporation, One Microsoft Way, Redmond, WA98052-6399, USA.

MWG-Biotech AG, Anzingerstr.7a, 85560 Ebersberg, Germany.

Norlab Instruments Ltd, Kirkhill Place, Kirkhill Industrial Estate, Dyce, Aberdeen, AB2 OES, UK.

Novus Biologicals, Inc, PO Box 802, Littleton, CO 80160, USA.

PeptaNova GmbH, Keplerstr. 26, 69207 Sandhausen, Germany.

Peptides International, Inc, PO Box 24658, Louisville, Kentucky 40224, USA.

PolyPeptide Laboratories, Halchtersche Str. 49, 38304 Wolfenbüttel.

Premier Biosoft international, 3786 Corina Way, Palo Alto, CA 94303-4504, USA.

R&D Systems, 4-10 The Quadrant, Barton Lane, Abingdon, OXON, OX14 3YS, UK.

Reichert Ophthalmic Instruments, 3362 Walden Ave, Depew, NY 14043, USA.

Roche Diagnostics Ltd, Bell Lane, Lewes, East Sussex, BN7 1 LG, UK.

Scie-Plas, Unit 3 Gainsborough Trading Estate, Old Road, Southam Warwickshire, CV47 HIP, UK.

Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK.

Stratagene Europe, Gebouw California, Hogehilweg 15 1101 CB Amsterdam Zuidoost The Netherlands.

Tocris Cookson Ltd, Northpoint, Fourth Way, Avon Mouth, Bristol, BSlI 8TA, UK.

Ultra-Violet Products Ltd, Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, CB4 1TG, UK.

Vector Laboratories, 30 Ingold Road Burlingame, CA 94010, USA.

VWR International, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, UK

Whatman PLC, Whatman House, St Leonards Rd, 20/20 Maidstone, Kent, ME16 OLS, UK.

URLs:

http://www .ncbi.nlm.nih.gov /entrez/

2.2 Animals and Tissues

2.2.1 Mice

Adult male CD-1 mice, aged 8 wk, were obtained from Harlan Olac (Bicester, UK). The mice were housed on receipt for 2 wk at 21°C with a 12:12-h light-dark cycle (lights on at 0700) and fed a commercial rodent diet (CRM Diet, Labsure, Witham, UK) containing 19.2% protein and 4.3% lipid (wt/wt). Both food and water were available *ad libitum*. The mice were killed by cervical dislocation, and the following tissues were rapidly removed and frozen in liquid nitrogen: muscle, small intestine, liver, kidney, brain and various white fat depots (gonadal, mesenteric, omental, and subcutaneous). The omental depot, which is small and can be hard to localise in lean mice, is located alongside the inferior surface of the stomach and is distinct from the mesenteric fat. All tissues were stored at -80°C until analysis.

2.2.2 Human tissues

Abdominal subcutaneous and omental WAT were provided by Dr S Wood (University of Liverpool) and were taken during gastroplasty from six obese male patients, aged 35-48 years with body mass index >33.5; the subjects did not exhibit any other ongoing diseases, such as infection or cancer. The studies were approved by Sefton Ethics Committee and all patients gave their informed consent. After removal, samples were immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Subcutaneous abdominal adipose tissue samples were also provided by Dr Mònica Bullò (Universitat Rovira I Virgili, Reus, Spain) and were obtained from 4 morbidly obese (BMI 40.1-49.3 kg/m^2) and 4 lean or slightly overweight (BMI 23.5-26.5 kg/m^2). Subjects' samples were obtained by biopsy or during surgery (cholecystectomy or abdominal hernia repair). The procedure of obtaining tissues and the study protocol were approved by the Ethics committee of the Sant Joan University Hospital (Catalunya, Spain); all subjects gave their informed consent. All samples were stored at -80°C until analysis.

2.3 3T3-L1 Cell Culture

2.3.1 Reagents

1. Culture Medium

   1xDMEM(25 mM glucose, 1.0 mM pyruvate, 4.02 mM L-alanyl-L-glutamine)

   10% FCS

2. Induction Medium

   1xDMEM

   10% FCS

   5 μg/ml insulin

   0.25 μM dexamethasone

   0.5 mM IBMX

3. Feeding Medium

   1xDMEM

   10%FCS

   5 μg/ml insulin

All media were prepared using a Corning filter systems (incorporating a 0.22 μm sterilising filter membrane) in a cell culture hood. Prepared media were pre-warmed to 37°C in a water bath prior to use and stored at 4°C at other times. Culture and feeding media were used for up to 2 weeks after preparation; however, induction medium was always freshly prepared prior to actual use and any excess discarded thereafter.

4. 1x PBS

   0.286 M sodium chloride

   5.55 mM potassium chloride

   16.4 mM disodium hydrogen phosphate

   2.94 mM potassium dihydrogen orthophosphate

   Autoclaved distilled water

5. Trypsin/EDTA

   Autoclaved distilled water

   0.005%/0.002% trypsin/EDTA

   1x PBS

2.3.2 Culture method

3T3-L1 cells were obtained from the American Type Culture Collection (Manassas,Viginia, USA) and aliquots were stored in liquid nitrogen. Cells were cultured at 37°C in an incubator with a humidified atmosphere of 5% CO_2 /95% air. Prior to each episode of cell culture work, the cell culture hood was thoroughly wiped with a 1% Virkon solution followed by 70% ethanol in order to maintain a sterile working environment; this wiping procedure was also performed after each episode of work was completed. All items were sprayed with 70% ethanol before being placed inside the hood, or packaging was opened, such that the sterile item within entered directly into the hood environment. Hands were sprayed repeatedly as necessary following removal from the hood and handling items outside of the hood.

To initiate cell culture, a frozen aliquot was removed from liquid nitrogen storage and thawed by placing it immediately into a water bath at 37°C. Prior to opening the cryovial inside a culture hood, it was thoroughly wiped with tissue sprayed with 70% ethanol to avoid contamination of the contents. One ml of pre-warmed culture medium was then added to the aliquot. The thawed cell suspension was transferred to a 25 cm^2 flask containing 3 ml of culture medium and placed in an incubator until the cells were ~80% confluent. The degree of confluency was checked using light microscopy. Cells were fed every 2-3 days with culture medium by aspirating the old media and replacing with 2 ml of fresh medium.

Once cells reached ~80% confluency (for 3T3-L1 cells, usually 5 days after being placed in the flask), the medium was removed and 1 ml of trypsin/EDTA was added to the flask which was then placed in the incubator for 1-2 min. The cells were then examined by light microscopy to establish that they detached once the flask was knocked gently against the bench. Nine ml of pre-warmed culture medium was added to the flask without delay (to neutralize the trypsin and prevent cell lysis) and then pipetted across the culture surface several times to wash off all the cells. The suspended cells were then pipetted up and down several times against the corner of the flask to break up any clumps.

For the original flask (generated from a thawed aliquot), cells were passaged into new flasks to generate sufficient numbers for experimental use and also to ensure a homogeneous and healthy cell population. One ml of the cell suspension was transferred to each new flask containing 3 ml of culture medium. Cells trypsinised from these flasks were subcultured out to 6- or 12-well plates as required by adding (respectively) 300 or 150 µl of the cell suspension to 1.5 or 1 ml of culture medium per well. Cells were fed every 2-3 days with culture medium until confluent.

Differentiation into adipocytes was examined under a light microscope by phase contrast at 100x magnification. The phase contrast image of the accumulation of lipid droplets during the development of adipocytes was captured and photographed (Fig 2.1).

Differentiation of the cells was initiated 24-48 h after they reached confluence by incubation for 2 days in freshly-prepared induction medium; this was followed by maintenance in feeding medium (renewed every 2-3 days).

Time course studies during 3T3-L1 adipocyte differentiation were performed by collecting media samples every 2 days; cells were harvested at the appropriate time points.

2.4 SGBS Cell Culture

2.4.1 Reagents

1. **Serum-containing medium** *(used in preadipocyte phase)*

   DMEM/Nutrient Mix F12

   33 µM Biotin

   17 µM Pantothenate

   100 units/ml Penicillin

   100 µg/ml Streptomycin

   10% FCS

2. **Feeding Medium** *(used throughout the renewal of medium)*

   DMEM/Nutrient Mix F12

   33 µM Biotin

   17 µM Pantothenate

100 units/ml Penicillin

100 µg/ml Streptomycin

10 µg/ml Human Transferrin

10 nM Insulin

100 nM Cortisol

200 pM T_3

3. **Induction Medium** *(used once when cells are confluent (day 0))*

DMEM/Nutrient Mix F12

33 µM Biotin

17 µM Pantothenate

100 units/ml Penicillin

100 µg/ml Streptomycin

10 µg/ml Human Transferrin

10 nM Insulin

100 nM Cortisol

200 pM T_3

250 nM Dexamethasone

500 mM IBMX

10 mg/ml Rosiglitazone

All media were prepared using a sterilizing filter system in a cell culture fume cupboard. The media prepared was stored in a fridge with a temperature of 4°C. Before use, the media has to be warmed up to 37°C in a water bath. The culture and feeding media prepared can be used for up to 2 weeks. The induction medium, however, must be made freshly immediately before use.

2.4.2 Culture Method

SGBS preadipocytes were kindly supplied by Professor Martin Wabitsch (University of Ulm, Germany). They are a human preadipocyte cell "strain" derived from the stromal-vascular fraction of the subcutaneous white adipose tissue of an infant with Simpson-Golabi-Behmel syndrome. The cell strain found shows a high capacity for

adipose differentiation, resulting in mature fat cells which are biochemically and functionally similar to human adipocyes (Wabitsch *et al.,* 2001).

A frozen SGBS cell stock in a cryovial was taken out from the liquid nitrogen and quickly defrosted in a 37°C water bath. To entirely thaw the cells, 1 ml of pre-warmed culture medium was added to the cryovial and gently pipetted up and down several times. The thawed cell suspension was then transferred to a 25 cm^2 flask containing 2 ml of culture medium. The cells were maintained in an incubator at 37°C in a humidified atmosphere with 5% CO_2/95% air. The culture medium was changed two to three times a week until 80%-90% of the cells were confluent. At this point, the cells were ready to be passaged and subcultured. Prior to trypsinisation, the cells were briefly washed once with sterilized 1 xPBS. Then, 1 ml of trypsin/EDTA was added to the flask and the flask immediately put in a 37°C incubator for 1 min to help quickly detach the cells. To examine whether the cells were trypsinised completely, the flask was checked under a light microscope. After the cells were entirely detached, 9 ml of culture medium was added in a final volume of 10 ml. The cell suspension was gently pipetted up and down for several times to mix cells evenly. Equal density of preadipocytes was seeded in new flasks, and 6-well or 12-well plates according to the experimental requirement. The culture medium was changed two or three times a week until cells were ready to be induced.

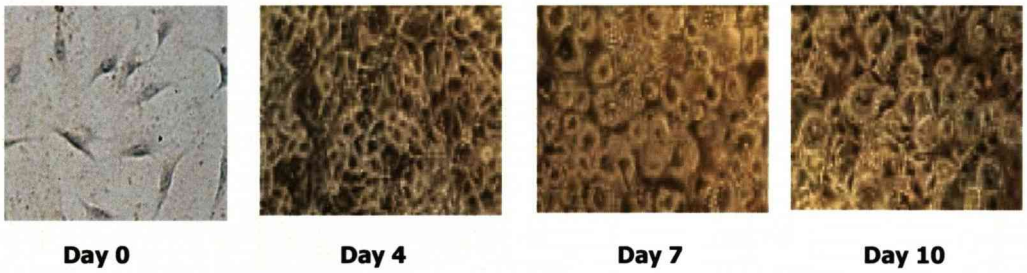
2.4.3 Differentiation of SGBS cells

Differentiation of the cells was initiated 24 h after 100% confluence in the plates. After aspiration of the old medium, the cells were gently washed with pre-warmed 1x PBS for three times, and then incubated for 4 days in the FCS-free medium containing 0.25 µM dexamethasone, 500 µM 3-isobutyl-1-methyl-xanthine, 10 nM insulin, 200 pM triiodothyronine (T_3), 1 µM cortisol and 2 µM rosiglitazone. Differentiation into adipocytes was examined under a light microscope by phase contrast at 100x magnification. The phase contrast image of the accumulation of lipid droplets during the development of adipocytes was captured and photographed by a CCD video camera (UVP Inc., California, USA) (Figure 2.1). More than 90% of SGBS cells underwent full differentiation into mature adipocytes after day 10 post-induction.

Fig 2.1 SGBS and 3T3-L1 cells cultured as *in vitro* models of human and mouse white adipocytes respectively

SGBS Human cell models

3T3-L1 Mouse cells models



The photographs shown above demonstrate the development of human SGBS and mouse 3T3-L1 adipocytes in culture medium at Day –3, Day 0, Day 6 and Day 20 or Day 0 Day 4 Day 7 and Day 10 respectively. Day –3 and Day 0 show a view of fibroblastic preadipocytes.

2.4.4 Harvesting the Cells

The cells were maintained in the feeding medium containing insulin, cortisol and T_3, which was renewed two or three times a week. According to the particular experimental design, the cells were collected at several time-points.

In the case of the time-course study of gut hormone receptor gene expression during differentiation, the cells were kept for up to 18 days and collected every 1-3 days in 700 µl of Trizol per well. Samples were stored at -80°C until analysis. The cells were maintained in the feeding medium for 11-15 days for the studies on the regulation of GIP, GLP-1, ghrelin and obestatin treatment(s) on expression of various adipokine genes.

2.4.5 Time-Course Cells

A plan was organized to collect the cells and their medium, which was done every two days accordingly (starting from day -3 up to day 18) as shown below:

-3 -1 0 1 2 3 4 6 8 10 12 15 18

Cells and their medium were collected in 1.5 ml tubes on each of these days (preferably at the same time) and stored in a -80°C freezer until required for use.

2.5 RNA preparation

2.5.1 Total RNA isolation from cells and tissues

2.5.1.1 Reagents

Trizol (Invitrogen)
Chloroform (Sigma)
Isopropanol (Sigma)
75% Ethanol (Sigma)
Ultra-Pure water (0.1 µm filtered, DNase free & RNase free)

2.5.1.2 RNA Extraction Method

Scissors, tweezers and electric homogenizer blade attachments were treated with RNAse AWAY and rinsed with UV-irradiated distilled water prior to use to remove

any RNAses present. If glass homogenizers were used, these were baked at 220°C for at least 2 h beforehand. For each group of samples, the glass homogenizer used was changed. If the electric homogenizer was used, the blade was rinsed twice with UV - irradiated distilled water between groups.

For RNA extraction from tissue, 50-150 mg of snap-frozen tissue were placed in a sterile tube. If necessary, the required quantity of tissue was cut from the frozen sample using a sterile scalpel, dissecting scissors and tweezers while on dry ice (alternatively, the tissue sample could be placed in a polythene sachet and broken into smaller pieces by hitting gently with a hammer). One ml of Tri-Reagent was added to the tube and the sample homogenized using either a glass or an electric homogenizer (Polytron Ultra-Turrax T25). The tissue homogenate was transferred into a 2 ml tube and centrifuged at 12,000xg for 10 min at 4°C. The clear supernatant was aspirated and transferred into a fresh 2 ml tube. Care was taken not to aspirate the cell debris pellet or any lipid layer overlying the supernatant. For RNA extraction from cells, the cells were first collected using Tri-Reagent as described and homogenised by triturating 10 times using a 1 ml syringe with a 23 gauge needle.

After incubating the tissue or cell homogenate at room temperature for 5 min, 200 µl of chloroform were added per ml of Tri-Reagent used. The tube was shaken vigorously for 15 sec by hand and then stood for 2-3 min at room temperature. This was followed by centrifugation at 12,000xg for 15 min at 4°C, leading to separation of three layers - a colourless upper aqueous phase containing RNA, a white interphase containing DNA and a red phenol phase containing protein. The aqueous layer containing the RNA was transferred into a fresh 2 ml tube, taking care to avoid contamination with either of the other two phases. If protein isolation was required the phenol phase was saved and stored at -20°C.

To minimise any possible genomic DNA contamination, an optional step (recommended by Sigma) was performed. Fifty µl of isopropanol were added to the sample per ml of Tri-Reagent used. This was vortexed, allowed to stand for 5 min at room temperature and then centrifuged at 12,000xg for 10 min at 4°C. The supernatant was transferred to a 1.5 ml tube, taking care not to aspirate in the area where any DNA pellet would have been precipitated.

Isopropanol was then added to the sample (450 µl per ml of Tri-Reagent used in sample preparation), followed by vortexing, incubation of the sample for 10 min at

room temperature and centrifugation at 12,000xg for 10 min at 4°C. After this step the precipitated RNA pellet could usually be identified at the base of the tube.
Pure ethanol was diluted with distilled water to prepare a 75% ethanol solution. The supernatant was poured off and 700 µl of 75% ethanol added to the RNA pellet. This was followed by vortexing and centrifugation at 10,000xg for 10 min at 4°C. The supernatant was again poured off, and any remaining volume aspirated by pipette and discarded. The RNA pellet was allowed to air dry for 2-3 min and then dissolved by adding 10-15 µl of ultra-pure water, then vortexing briefly and centrifugating. Samples could be stored temporarily if required at -20°C prior to DNase treatment and/or quantification. Scissors, tweezers and glass homogenizers or electric homogenizer blade components were soaked in 1% Virkon solution for 1-2 h to decontaminate, then cleaned of any tissue debris prior to rinsing.

2.5.2 DNAse treatment of RNA

This treatment to further minimise any genomic DNA contamination was performed for all RNA samples that were to be analysed using real-time PCR. The efficacy of the treatment was established by DNase treating aliquots of two RNA samples known to contain genomic DNA. Treated and untreated RNA aliquots were then subject to PCR using a primer pair known to generate a band of a specific size if genomic DNA was present in the template. To check that the DNase treatment did not degrade the RNA, the treated and untreated samples were reverse-transcribed, and PCR performed using the cDNA.

2.5.2.1 Reagents

DNA-free Kit:

- 10x DNase I buffer
- 2 units/ µl DNase (RNase free)
- DNase inactivation reagent

2.5.2.2 Method

One µl of DNAse I and 2.5 µl of 10x DNAse I buffer were added to a 25 µl RNA sample, gently vortexed and incubated at 37°C in a hybridisation oven (HB-1000) for 20-25 min. Five µl of DNAse inactivation reagent were added to the tube and vortexed gently. This was followed by an incubation step of 2 min at room temperature, flicking the tubes once during the incubation to redisperse the DNAse I inactivation reagent.

The sample was centrifuged at 10000xg for 1 min at room temperature to pellet the DNAse inactivation reagent and then stored temporarily if required at -20°C until quantification. Transfer of the RNA solution into a new 1.5 ml tube was usually not necessary, as the sample could be centrifuged prior to use so that the DNase inactivation reagent pellet was not disturbed when removing aliquots of RNA.

2.5.3 RNA Quantification

The RNA extracted samples were passed through a spectrophotometer machine (BioPhotometer) to read and calculate their concentration in µg/µl. First, the extracted RNA has to be diluted. This is done by adding 1µl of the RNA to 69µl of pure water to new tubes to give a 1 in 70 dilution. Then, 70µl of pure water was added to a cuvette (spectrophotometer special sterile tube) to act as a blank. The RNA tubes were measured straight away making sure that the cuvette was rinsed with water before each use. The RNA concentration was determined from the absorbance at 260 nM (Yao *et al.,* 2004), which has a coefficient number of 44. To determine the concentration of RNA; the Beer-Lambert law was used.

The Beer-Lambert law:

[RNA] µg/µl = A260 x 44 x 70 / 1000

2.5.4 RNA integrity

RT-PCR was performed using the isolated RNA samples. Once the cDNAs had been synthesised, their quality was checked by performing PCR using primers for the housekeeping gene β-actin and running the products on a gel. The band intensity indicated the quality of the cDNA sample from which the integrity of the original

RNA template could be inferred. Faint or undetectable bands suggested degradation of the isolated RNA or a failed RT reaction. The former could be investigated by checking the RNA integrity directly, and if found to be significantly degraded the sample was re-extracted from the tissue.

mRNA comprises 1-5% of a total RNA sample, whereas ribosomal RNA (rRNA) makes up >80% of total RNA, the majority of that consisting of *28S* and *18S* rRNA species (in mammals). The method used to directly check RNA integrity (denaturing gel electrophoresis) relies on the assumption that rRNA quality and quantity reflect that of the underlying mRNA population, with the caveat that there may be a degree of difference between the integrity of the longlived and abundant rRNA molecules and that of the underlying mRNA population, which turns over much more rapidly (Palmer & Prediger, 2005).

2.5.4.1 Reagents

Ultra-pure water
Sample buffer (1 ml 40% glycerol, 40 µl saturated BPB solution)
Reagents for agarose gel electrophoresis

2.5.4.2 Method

One µg of RNA was diluted with ultra-pure water to a total volume of 5 µl in a 0.2 ml tube and 2 µl of sample buffer added. The sample was mixed by vortexing briefly, loaded onto a 1 % agarose gel and run at 80-100 V for 30-40 min. An image of the gel was photographed. An intact RNA sample would have two clearly visible and well-defined bands representing 28*S* and 18*S* rRNA with little or no visible debris at the base of the gel. RNA degradation would be inferred from smudging or absence of the rRNA bands and a strong signal at the base of the gel.

2.6 Reverse Transcriptase Polymerase Chain Reaction (RT PCR)

2.6.1 Reagents

Reverse-iT 1st Strand Synthesis Kit (ABgene):

- Anchored Oligo-dT

- 5x 1st Strand Synthesis Buffer
- 5 mM dNTP mix (each)
- Reverse-iT Rtase Blend
- DTT
- Pure RNA water

2.6.2 Method

Each tube had 1 µg of RNA and 1 µl of Anchored Oligo-dT, which is diluted with pure water to 12 µl. The tubes were incubated at 70°C for 5 minutes to denature the RNA. After that, the tubes were placed on ice to prepare the master mix. An RT-PCR standard master mix was prepared. Each tube contained 4µl of the buffer, 2µl of the dNTP, 1 µl of Reverse-iT RTase and 1 µl of DTT. So, for 12 samples, 48 µl of the buffer, 24 µl of the dNTP, 12µl of Reverse-iT RTase and 12 µl of DTT were prepared.

The mixture was vortexed and pelleted down using a centrifuge. Then it was incubated at 47°C for 30 min to stretch the reverse transcription of the RNA along the cDNA. An incubation of 75°C for 19 min followed, which is performed to denature the RTase. Again, the mixture was pelleted down prior taking for PCR (if not, it was stored at -20°C).

2.7 Polymerase Chain Reaction (PCR)

The PCR generates multiple copies of a cDNA. A master mix was prepared that includes the DNA, which was placed in a PCR machine. Firstly, the temperature was increased to 94°C, which causes the DNA double helix to separate (denaturation). Secondly, the temperature was lowered, which causes the primers to anneal (attach) to the double strand DNA. Primers are specific RNA segments that match to specific DNA sequences, which initiate the process of making new DNA. Thirdly, an enzyme called TAQ polymerase (ready mix) was used to make new DNA chains. The temperature was again increased and the enzymes made new DNA to match the DNA strands that were separated. The cycles were repeated many times; after only 20 cycles, there would be a million copies of the original DNA.

2.7.1 Reagents

- Ultra-pure water
- 10 μM each sense and antisense primer mix
- ReddyMix PCR Master Mix Kit:
  1.1xReddyMix

2.7.2 Method

This procedure was carried out using sterile 0.2 ml RNA/DNA-free tubes and filter tips and reactions were performed using a thermal cycler (PCR Express). A master mix containing 11.25 μl of 1.1x ReddyMix, 0.25 μl of primer mix and 0.5 μl of ultra-pure water per sample was prepared, vortexed and centrifuged. Twelve μl of this master mix were transferred into each tube and 0.5 μl of the appropriate cDNA added for a 12.5 μl final reaction volume (all volumes proportionately scaled up for a 50 μl reaction); ultra-pure water was used for the 'no template' control. The tubes were vortexed, centrifuged and placed into the cycler with the PCR program set as follows:

94°C, 2 min (denaturation)
20-38 cycles (specified cycle number for each primer pair) of:
i. 94°C, 20 sec (denaturation)
ii. 53-62°C, 30 sec (annealing; T_a optimized for each primer pair)
iii. 72°C, 30 sec (extension)
72°C, 5 min (final extension)

2.7.3 Primer Design and Optimisation

Primers were designed using Primer Premier 5 software and synthesized commercially (MWG Biotech, Ebersberg, Germany or Eurogentec, Romsey, UK). Primers were created by first pasting the mRNA coding sequence of the gene of interest into the software. This sequence was obtained from the NCBI PubMed Entrez Nucleotide database *(www.ncbi.nlm.nih.gov/entrez)* and the most complete and up-to-date sequence of those available for the gene was selected. The locations on the

mRNA coding sequence of any exon-exon boundaries, together with the start and end of the protein coding sequence (CDS) were identified.

Knowing the location of exon-exon boundaries was important as the primer pair could then be designed to span at least one intron in order to avoid the generation of PCR products from any genomic DNA contaminants present in the cDNA template solution. Intron locations were either established directly (for example using NCBI's Map Viewer utility) or calculated from the chromosomal exon locations when the sequence was subjected to a BLAST search against the genome. Ideally, the primers would be sited so as to generate a product from the CDS, but if it was necessary, one of the primers could be sited outside this region in order to span an intron.

Primers were designed according to several parameters. The primer length was chosen to be between 18 and 24 bases. Longer primers are more specific during amplification (allowing use of a lower T_a) but are more likely to form secondary structures including hairpins, dimers and self-complementation. If unavoidable, primers with the most unstable secondary structures were selected, as these structures would tend to disappear at a higher T_a. Dimers, or false priming sites involving the 3' end of the primer, were especially avoided as these could theoretically be extended by the DNA polymerase.

Ideally, the primer melting temperature (T_m) was between 60-65°C (and within 2°C of each other for the pair), so that the T_a would not be below 55°C (T_a of a primer pair should be ~5°C lower than the average of their T_ms; a low T_a promotes nonspecific binding). The guanine-cytidine (GC) content of the primers was generally 40-60%, and roughly equal between the two primers, as with increasing GC content the T_m increases. The amplicon size was ideally 180-600 bp; larger products would require longer annealing times in order to be fully extended. The primer sequences as well as the expected amplicon sequences were subjected to a BLAST search to ensure specificity prior to ordering.

Primers were tested on receipt by performing PCR with one or more cDNA samples in which a signal for that gene might be expected. A high cycle number (38 or more) and a low annealing temperature (53-55°C) were used initially to maximise the

chance of obtaining a product. The optimal T_a of a primer pair was determined by performing PCR over a range of annealing temperatures and selecting the highest temperature at which a strong signal was obtained with no non-specific products. The cycle number was optimized by performing PCR over a range of cycle numbers and selecting the lowest cycle number for which a distinct signal could be detected. Primer sequences and optimal cycling conditions are detailed in Table 2.1.

2.8 Agarose gel electrophoresis

2.8.1 Reagents

- Agarose
- 5 mg/ml ethidium bromide
- 10x TBE buffer
- 100 bp DNA ladder (GeneRuler or ReddyRun Super-ladder low 100 bp)
- Distilled water

❖ Distilled water was used to dilute l0x TBE buffer to prepare 1x TBE buffer for use.

❖ 1 % agarose gel (final volume 50/60 ml):

0.5/0.6 mg agarose

50/60 ml lx TBE buffer

5/6 µl ethidium bromide (final concentration 0.5 µg/ml)

2.8.2 Method

The gel casting tray was prepared by sealing the ends with autoclave tape. The agarose was weighed directly into a 500 ml flask, the 1x TBE added and the flask heated for ~2 min in a microwave (flask taken out and swirled every 30 sec) until the agarose was completely dissolved. The solution was cooled by running the flask under cold water prior to adding the ethidium bromide, which was mixed by swirling the flask. The mixture was poured into the gel casting tray and any bubbles pushed

Table 2.1 Sequences of PCR primers

Primer	Ta (°C)	Cycles	Product (bp)	Sequence (5'- 3')
mβ- actin s	60	20	463	TGCTGTCCCTGTATGCCTCT
mβ-actin as				AGGTCTTTACGGATGTCAACG
mResistin s	60	30	266	GAAGAACCTTTCATTTCCCCTCC
mResistin as				CTTCACGAATGTCCCACGAGGC
mGLP-1R s	59.9	36	328	TCCTCCGAGAGCACTGTCCGTCTT
mGLP-1R as				CAGCAGCCCTCGTCCTCATAGA
mGIPR s	57.5	36	489	TATGCCTGCTGGAACTACACGG
mGIPR as				GGTACGGAAATAGATGGGAAACG
mGHSR s	63.3	36	217	TGGAGCACGAGAACGGCACAGA
mGHSR as				GGGCAGCCAGCAGAGGATGAAA
mGPR39 s	55	35	355	GTCAGTTTGGCTTGTTCAGATA
mGPR39 as				AGTTGAGTCCCTTGTGAGTGG
hβ- actin s	56.2	28	281	GTGGCATCCACGAAACTACCTT
hβ-actin as				GGACTCGTCATACTCCTGCTTG
hLeptin s	55	38	422	GAACCCTGTGCGGATTCTTG
hLeptin as				CACCTCTGTGGAGTAGCCTGAA
hAdiponectin s	60	31	352	ATGCTGTTGCTGGGAGCTGTTC
hAdiponectin as				CCACACTGAATGCTGAGCGGTA
hGLP-1R s	62.5	36	268	GCCGCCTGGTGTTTCTGCTCAT
hGLP-1R as				GCGTGCTCGTCCATCACAAAGG
hGIPR s	61	36	448	TATCCGCATTCTTGGCATTCTCC
hGIPR as				CTCCGACTGCACCTCCTTGTTG
hGHSR s	55.8	36	320	CGCTCAGGGACCAGAACCA
hGHSR as				GGCCCGAGAACTTTCATCTTTC

hGPR39 s				CAGCCCGAGACCTCCAATA
	58	33	453	
hGPR39 as				AAACTGCTGCGAGGACACC

This table shows the sequences of the primers used in PCR analysis. The PCR product sizes, the annealing temperatures (T_a) and amplification cycle numbers are those used unless otherwise indicated. β-actin was used as a housekeeping gene. m, mouse; h, human; s,sense primer; as, anti sense primer.

aside using a comb. The combs were then placed into their slots and the gel allowed to set for at least 15 min. The autoclave tape was removed and the tray with the gel placed in the gel tank. The tank was filled with 1 x TBE until the gel was submerged in the buffer before removing the combs.

For analyzing the PCR products on the gel, 10 µl of each was pipetted sample into separate wells. Two µl of ladder was loaded near the samples as a reference to assess whether the PCR product was located at the expected size. The end wells were avoided if possible, as these are most likely to run aberrantly. The gel was run at 85-100 V for 30-60 min or until the samples had run far enough. For band detection, the gel was placed on a UV transilluminator (2011 Macrovue). The images were recorded using a DC120 digital camera and analyzed using ID 1 image analysis software.

2.9 PCR product purification and sequencing

Correct primer design should ensure that the product obtained with PCR is the intended one. However, in order to confirm that the target cDNA has indeed been amplified, it is important to sequence the product obtained from the use of a new primer pair. The sequencing was performed commercially by MWG Biotech. (Table 2.2)

2.9.1 Reagents

- ReddyMix PCR Master Mix Kit (1.1x ReddyMix)
- NucleoSpin Extract 2-in-l Kit:
  - Buffer NTl
  - Buffer NT2 Buffer NT3
  - NucleoSpin columns
  - NucleoSpin collecting tubes
- Ultra-pure water
- 100bp ladder GeneRuler
- Sample buffer (1 ml 40% glycerol, 40 µl saturated BPB solution)

2.9.2 Method

PCR reactions using a single template cDNA, previously identified as strongly expressing the gene of interest, were performed to a total reaction volume of 100 µl

Table 2.2 PCR product sequencing

Best Hits from Blast of

Passed Sequences vs. Non-Redundant Protein Database

FAQ on Blastx Best Hits Report

	Sample Name (Query	Query Region	DNA +/-	DatabaseEntry (Subject)	Subject Region	Description	Scorse	E value
1	GLP-1f-GLP-p	2..292	+	gi|565107| Length 463	279..375	Glucagon-like peptide 1 (7-36) amide receptor, glucagon-like peptide 1 (7-36) amide receptor, GLP-1(7-36) amide receptor, GLP-1 receptor [Human, peptide, 463aa]279375	202 100% identity in 97aa	3e-51
2	GIP-f-GIP-p	34..177	+	gi|1785516| Length 466	351..398	Gastric inhibitory polypeptide receptor [Homo sapiens] ref|NP-000155.1| Gastric inhibitory polypeptide receptor[Homo sapiens] gb|AAC97984.1| GIP receptor [Homo sapiens] gb|AAA84418.1	27 95% identity in 48aa	4e-21
3	GHSRf-GHSRp	3..338	+	gi|11931795| Length 412	285..396	Homo sapiens growth hormone secretagogue receptor (GHSR), transcript variant 1a, mRNA GHSR-f- GHSR-p2e-133 [Homo sapiens] growth hormone secretagogue receptor (GHSR), transcript variant 1b, mRNA		5e-42
4	GPR39-f-GPR39-p	1..366	+	gi|4504097| Length 453	224..345	G protein-coupled receptor 39[Homo sapiens] sp|o43194|GPR39_HUMAN probable G-protein coupled receptor 39 gb|AAC26082.1| GPR39 [Homo sapiens] 224 345	245 100% identity in 122aa	2e-64

This table showes the PCR product sequencing in order to confirm that the target cDNA has been amplified. This was performed commercially by MWG Biotech.

(2 x 50 µl reactions) and using a high number of cycles (38-40) in order to generate a large quantity of the PCR product. After cycling, the reaction volumes were pooled and loaded onto a 1 % agarose gel, using a single composite well (obtained by taping several teeth of the comb together in advance). The gel was run at 100 V for 30 min.

The band was examined under low-wavelength UV light, excised with a clean scalpel and the gel fragment transferred to a 2 ml tube and it's mass determined. The PCR product was extracted from the gel using the standard protocol for the NucleoSpin Extract 2-in-1 kit as follows:

For each 100 mg of the gel fragment, 300 µl of NT1 was added. A water bath was used to incubate the tube at 50°C until the gel had dissolved (~10 min) and was accompanied by vortexing the tube briefly every 2 min. A column was placed into a collecting tube, 700 µl of the sample loaded and centrifuged for 1 min at 8000xg.

The flow-through in the collecting tube was discarded. If the sample volume was above 700 µl, this procedure was repeated using further aliquots of up to 700 µl each time until the total volume had been processed. As a result of this step, the (impure) PCR product was bound to the silica membrane of the column. To remove inhibitors and contaminants resulting from the agarose, a recommended washing step was performed by adding 500 µl of NT2 to the column and centrifuging for 1 min at 11000x*g*. The flow-through was discarded, 600 µl of NT3 added and the column centrifuged for 1 min at 11000x*g* to wash the membrane. The flow-through was again discarded, 200 µl of NT3 added and the column centrifuged for 2 min at 11000x*g* to dry the membrane. The column was then placed into a 1.5 ml tube and 30 µl of ultra-pure water added. The column was allowed to stand for 1 min at room temperature to allow full dissolution of the DNA. This was followed by centrifugation for 1 min at 11000xg to collect the eluted DNA in the 1.5 ml tube.

In order to be accurately sequenced, 20 ng of DNA are needed per 100 bp of the product size. The concentration of the purified DNA solution was estimated by preparing a sample consisting of 1 µl of the purified DNA, 5 µl of ultra-pure water and 2 µl of sample buffer. This was loaded into a well of a 1 % agarose gel and run as described above alongside 2 µl of quantifying 100 bp ladder (GeneRuler). According to the brightness of the purified DNA band, the concentration of the solution could be estimated as described below. If the band was extremely faint or absent, the whole procedure was repeated from the PCR reaction onwards.

The 100 bp GeneRuler ladder was prepared using 1 µl (0.5 µg) of ladder DNA, 1 µl of loading dye solution and 4 µl of ultra-pure water. The DNA was therefore diluted 1:6 in the final preparation (0.083 µg/ µl). Two µl of ladder, containing 0.17 µg of DNA, were loaded onto the gel. According to the manufacturer's protocol a specific quantity of DNA is present in each band when 1 µg of the ladder DNA is used. Thus, the amount of DNA present in each band when 0.16 µg of ladder are used could be determined.

When 1 µl of the purified DNA solution was run against the ladder, the brightness of the purified DNA band was compared to that of each of the ladder bands by eye and the nearest match identified. The DNA concentration of that ladder band was then taken to be the approximate concentration of the purified DNA solution. An aliquot of this solution containing the amount of DNA required for sequencing was then dried in a 1.5 ml tube using a vacuum centrifuge. This was then sent for sequencing together with a 10 µl aliquot of a 10 µM solution of either the sense or the antisense primer (to sequence both the sense and antisense strands, two DNA aliquots were sent together with an aliquot of each primer). The sequencing result was subjected to a BLAST search to confirm identity.

2.10 Real-time polymerase chain reaction

Real-time PCR can be used for absolute or relative quantitation of the initial amount of template in a sensitive, specific and reproducible manner. It is preferable to alternative forms of PCR quantification because it quantifies the initial template cDNA rather than the final amount of amplified PCR product. This is possible as real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle. The fluorescent signal increases in direct proportion to the amount of PCR product. During the exponential phase of the PCR reaction, there is a significant increase in the amount of PCR product. By monitoring the fluorescence, the cycle at which this phase commences can be determined. The more initial template there is, the earlier the fluorescent signal will be significantly increased.

Real-time PCR also offers a wide dynamic range of up to 10^7 -fold, compared to only 10^3 -fold in conventional PCR, which means that large variations in template amount can be quantified sensitively and accurately.

2.10.1 Taqman® system

The real-time PCR work performed in this study employed the Taqman system (Applied Biosystems), which involves the use of a primer pair and a Taqman probe. The probe is an oligonucleotide with a fluorescent (reporter) dye (usually FAM) on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base. When the probe is intact, energy is transferred from the reporter dye to the nearby quenching dye molecule (FRET - fluorescence resonance energy transfer) rather than emitted as fluorescence (Giulietti *et al.,* 2001). During the annealing phase of the PCR cycle, the probe binds to an internal region of a PCR product and when the Taq polymerase replicates a template onto which a probe is bound, its 5' exonuclease activity cleaves the probe. This separates the reporter and quenching dyes and ends the FRET. The reporter dye now starts to emit fluorescence (at 518 nm for FAM) which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence (ΔR) of the reporter dye (usually plotted as ΔR_N after normalisation of the reporter dye signal to a reference dye (usually ROX) to correct for differences in reaction mix volumes between wells) (Giulietti *et al.,* 2001).

2.10.2 Design of primers and Taqman probes

Human IL-1β primers and Taqman probe sequences were designed by Primer Express software and synthesised commercially (Eurogentec for primers and probes). Sequences and intron data were obtained as in section 2.7.3, and primers were again designed to span an intron. Primers and probes were designed to be 20-30 bases in length, with primer T_ms of 58-60°C and the probe T_m 10°C higher. The product size was designed to be 50-150 bases as PCR is more efficient with smaller amplicons. One specific requirement for the Taqman probes was that there be no G at the 5' end, as a G adjacent to the reporter dye quenches reporter fluorescence even after the probe

Table 2.3 Real-time PCR primer and Taqman probe sequences

Primer/Probe	Product (bp)	Final conc. (nM)	Sequence (5'-3')
qhB-actin s		900	TTGCCGACAGGATGCAGAA
qhB-actin as	101	900	GCCGATCCACACGGAGTACT
qhB-actin P		200	AGCACAATGAAGATCAAGATCATTGCTCCTCCT
qhLeptin s		300	CCAAAACCCTCATCAAGACAATT
qhLeptin as	93	300	AGTCCAAACCGGTGACTTTCTG
qhLeptin P		250	TGACATTTCACACACGCAGTCAGTCTCCT
qhAdiponectin s		300	CCCAAAGAGGAGAGAGGAAGCT
qhAdiponectin as	73	300	TCCTTTCCATCATAGTAATATTTCCAGAA
qhAdiponectin p		200	TTCCCAGATGCCCCAGCAAGTGTAAC
qh IL-6 s		300	GGTACATCCTCGACGGCATCT
qh IL-6 as	83	300	GTGCCTCTTTGCTGCTTTCAC
qh IL-6 p		200	TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT
qh MCP-1s		300	CATAGCAGCCACCTTCATTCC
qh MCP-1as	104	300	TCTGCACTGAGATCTTCCTATTGG
qh MCP-1p		200	CAGCCAGATGCAATCAATGCCCC

This table shows the sequences of the primers and Taqman probes employed in real-time (q)PCR analysis for (relative) quantitation of gene expression.; h, human; s, sense primer; as, antisense primer; P, Taqman probe

has been cleaved. All other human primers and Taqman probes were kindly provided by Dr B Wang (University of Liverpool).

Primer and Taqman probe sequences are detailed in Table 2.3, together with the optimised final reaction concentrations. For a new primer/probe set, optimal primer concentrations were determined by performing an assay with final concentrations of sense and antisense primers ranging from 50 nM to 900 nM, in all possible combinations, with the probe concentration fixed at 200 nM. The resulting amplification plots were then examined and the combination with the lowest C_T and, the highest ΔR_N was selected. Taqman probe optimisation was then performed at concentrations from 25-225 nM using the optimised primer concentrations and the amplification plots were used to select the optimal probe concentration.

2.10.3 Preparation of 96 well plates for real-time PCR

2.10.3.1 Reagents

qPCR Core Kit:

10x reaction buffer

50 mM magnesium chloride

5 mM dNTP mix

5 U/µl Hot Goldstar enzyme

Sense primer, antisense primer and Taqman probe stock solutions

Ultra-pure water

2.10.3.2 Method

Working solutions of the primers and probes were prepared at 10x concentration (e.g. 9000 nM for a final reaction concentration of 900 nM) by diluting the stock solution with ultra-pure water. A master mix was made up which contained all the necessary components for the PCR, minus the cDNA template as follows:

l0 x reaction buffer	2.5 µl per well (1x final concentration)
50 mM MgCl	2.5 µl (5 mM)
5 mM dNTP	1 µl (200 mM)
Sense primer	2.5 µl (SEE TABLE (2.3))

Antisense primer	2.5 µl (SEE TABLE (2.3))
Taqman probe	2.5 µl (SEE TABLE (2.3))
5 U/µl Hot Goldstar enzyme	0.125 µl (0.025 U/µl)
Ultra-pure water	11.375 µl

Once the master mix had been made up, 25 µl were aliquoted to each well. One µl of the appropriate template cDNA was then added to each well for a total reaction volume of 26 µl and the plate sealed with an optical cover. All samples were run in duplicate, or triplicate if practical. No primer and no template controls were included on the plate for each set of primers and probe. The plate was spun at 1000 rpm for 20 sec to ensure the reaction mix was not sticking to the well sides. If the assay was not to be performed immediately, the plate could be stored for up to 5 days at 4°C. If a set of samples (comprising several groups) required analysis over several plates, at least one group (usually the control group) was present on all plates. Data were normalised such that the mean ΔC_T for the control group had the same value for each assay, enabling the data on the different plates to be directly compared.

2.10.3.3 Taqman system real time PCR setup

Reactions were performed using an Mx3005P cycler (Stratagene). Default conditions were used except that the reaction volume was specified as 26 µl. Once the cover temperature had reached 105°C, the plate was inserted into the heat block and an optical pad placed on top so that the holes aligned with the well positions. The cover was screwed into position and the run started.

Amplifications were performed commencing with a 2 min activation stage at 50°C, then a 10 min denaturation stage followed by 40 cycles consisting of a denaturation step of 15 sec at 95°C and a combined primer annealing and extension step for 60 sec at 60°C. Data were collected automatically by the software, and analysed once the run was complete (total run time was ~2 h).

2.10.3.4 Analysis of real-time PCR data

The amplification plots were displayed in log scale using the Sequence Detector software and the threshold manually adjusted to ensure that it was clear of any noise.

The software automatically recalculated the C_T value for each well (C_T defined as the cycle number at which the fluorescence emitted from the well crossed the threshold). The results were exported as a Microsoft Excel file and gene expression analysed by relative quantitation with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). All samples were normalised to values of β -actin, the difference of C_T values between the control and treated samples were calculated and the relative fold changes obtained between the two groups. The formulae used for calculation are as follows:

$\Delta C_T = C_T$ (Target gene)- C_T (Reference gene)

$\Delta\Delta C_T$ = Mean ΔC_T (treated samples) - Mean ΔC_T(control samples)

Fold Change=$2^{-\Delta\Delta CT}$

2.11 Western blotting

This technique enables the detection of a specific protein within a sample using a specific primary antibody. The protein samples are initially denatured using heat, SDS (to destroy hydrogen bonds and ionic interactions) and β-mercaptoethanol (to reduce disulphide bonds) to generate proteins with a linear structure. SDS also binds to the proteins (1.4 g of SDS per 1 g of protein) so that mass and charge are in equal proportion. The samples can then be separated according to their size by gel electrophoresis.

The negatively charged proteins are transferred from the gel to a membrane by electroblotting and a specific primary antibody used to bind with the antigen binding (Fab) part of the protein. A secondary antibody is used to bind to the constant region (Fc) of the primary antibody. The Fc of the secondary antibody is labeled with horseradish peroxidase (HRP); which in the technique employed here breaks down a chemiluminescent substrate in the presence of hydrogen peroxide and alkaline conditions. This chemiluminescent reaction emits light for up to 1 h. The maximum emission is at 428 nm and is detected by exposure of the membrane to a blue-light sensitive autoradiography film such as Hyperfilm ECL.

2.11.1 Protein isolation

2.11.1.1 Protein isolation from snap frozen tissue

2.11.1.1.1 Reagents

SHE buffer:

250 mM sucrose

1 mM HEPES

0.2 mM EDTA

Distilled water

Buffer PH adjusted to 7.2 and stored at 4°C.

2.11.1.1.2 Method

Between 100 and 200 mg of tissue were homogenized in 300-500 µl of SHE buffer using glass homogenisers, followed by centrifugation at 12000xg for 15 min at 4°C. The supernatant containing the protein was retrieved and the pellet discarded.

2.11.1.2 Protein isolation with Tri-Reagent

2.11.1.2.1 Reagents

Tri- Reagent

0.3 M guanidine hydrochloride in 95% ethanol (0.3M GHCl)

100% ethanol

1% sodium dodecyl sulphate (SDS)

2.11.1.2.2 Method

As described in section (2.5.1.2), the phenol layer obtained after phase separation with Tri-Reagent was retained to perform protein extraction. The sample was mixed by inversion with 300 ml of 100% ethanol, allowed to stand for 3 min at room temperature and then centrifuged at 2000xg for 5 min at 4°C to pellet any remaining DNA. The supernatant containing the protein was transferred into 2 ml tube and 1.5 ml isopropanol added to precipitate the protein.

The sample was allowed to stand for 10 min at room temperature and was then centrifuged at 12000xg for 10 min at 4°C. The protein was washed by discarding the

supernatant, adding 2 ml of 0.3 M GHCl and standing for 20 min at room temperature before centrifuging at 7500xg for 5 min at 4°C. This washing step was repeated twice more. After the final wash the protein was vortexed in a 2 ml of 100% ethanol and incubated for 20 min at room temperature. This was followed by centrifuging at 7500xg for 5 min at 4°C. The supernatant was discarded and the protein dried for 5 min under vacuum. The protein pellet was dissolved in 50-100 µl of SDS using a sonicator and stored at -80°C.

2.11.2 Protein quantification by BCA methods

The protein concentration was detected by the bicinchoninic acid (BCA) method. This assay involves initially the formation of a Cu^{+2} -protein complex under alkaline conditions, which is followed by the reduction of Cu^{+2} to Cu^{+1}, and then combination with BCA to form a complex, which is a purple colour and has an absorption maximum at wavelength of 570 nm. The raw data were collected and calculated using the Microplate Manager 5.2 software (Bio-Rad).

2.11.2.1 Reagents

- Bicinchoninic acid (BCA)
- Copper (II) sulphate solution
- Bovine serum albumin standard 2mg/ml(BSA)
- 1% SDS
- 96-well microplate

2.11.2.2 Method

A standard curve was prepared with final protein concentrations of 0, 4, 8, 12, 16, 20 and 40 µg/well. This was performed by adding 0, 2, 4, 6, 8, 10 and 20 µl of protein standard in duplicate to the wells and making the volume up to 20 µl with 1% SDS. One µl of each sample was added in duplicate to the plate and 19 µl of SDS added to a final volume of 20 µl in each well. A 1:50 dilution of copper sulphate in BCA was prepared. Two hundred µl of this solution were added to each well. The plate was incubated for 2 h at room temperature and the extinction measured at 570 nm with a

plate reader. Data were collected with Microplate Manger software, a standard curve was made and the protein concentrations of the samples determined.

2.11.3 SDS-PAGE

2.11.3.1 Reagents

1) Loading buffer (aliquots stored at -20°C)
   - 125 mM tris-HCl
   - 2.5% SDS
   - 2.5% β-mercaptoethanol
   - 6.25% glycerol
   - 2.5 mg/ml BPB
2) Separating gel (10 ml) (8%/12%)
   - 5.275/4.3 ml distilled water
   - 2.025/3 ml 40% acrylamide/bis
   - 2.5ml 1.5 M tris (pH 8.8)
   - 100µl 10% SDS
   - 100µl 10%AP
   - 6/4 µl TEMED
3) 4% Stacking gel (5 ml)
   - 3.732 ml distilled water
   - 498 µl 40% acrylamide/bis
   - 630 µl 1.0 M tris (pH 6.8)
   - 50µl 10% SDS
   - 50µl 10%AP
   - 5 µl TEMED
4) Running buffer
   - 25 mM tris-base
   - 192 Mm glycine
   - 0.1% SDS
   - Distilled water
5) Transfer buffer (pH 8.3)
   - 25 mM tris-base
   - 192 mM glycine
   - 20% methanol

Distilled water

Rainbow recombinant protein molecular weight markers (10-250 kDa)

Water- saturated n-butanol

2.11.3.2 Method

To run the gel a vertical electrophoresis unit was used. Prior to use the glass plates and spacers were cleaned with ethanol, dried and then assembled. The separating gel was inserted between the glass plates using a 5 ml syringe and 21 gauge needle to prevent the development of bubbles. A few drops of n-butanol were pipetted on top of the gel to stop it drying out and to ensure that it was level. Once the separating gel had set (~30 min) the n-butanol was removed with filter paper and a 12-well (30 µl/well) comb inserted. The stacking gel was then poured using a needle and syringe between the glass plates and allowed to set (~20 min). Between 5 and 20 µg of protein were used as required. The samples were diluted 1:1 with sample buffer and heated at 95°C for 5 min. The gel apparatus was transferred into the gel tank which was filled with 1.5 l of running buffer until the top of the wells were fully submerged. The comb was removed and the samples and 5 µl of protein marker were loaded into separate well. A maximum volume of 30 µl per well could be used. The gel was run for 1 h at 100 V or until the end of the run when the BPB line started to run off the gel. The electrophoresis unit was then disassembled and the gel transferred for electroblotting.
The blotting procedure was performed using compact electroblotting units. Two foam pads, a nitrocellulose membrane and two squares of extra thick blotting filter paper were pre-soaked in transfer buffer. The blotting unit was assembled as follows:

- White cassette clamp +ve side
- Pre- soaked foam pad
- Extra thick blotting paper
- Pre-wetted transfer membrane (Hybond ECL)
- Gel
- Extra thick blotting paper
- Pre-soaked foam pad
- Black cassette clamp –ve side

When the gel was laid on the membrane it was checked that no bubbles were trapped. The blotting unit was transferred into the blotting tank with the –ve

cassette clamp facing the –ve side of the tank. The tank was filled with transfer buffer until the membrane-gel assembly was fully submerged. The transfer was performed at 100 V for 1 h and the system was cooled with water during the run.

2.11.4 Immunological detection of proteins

2.11.4.1 Reagents

1) Tris buffered saline (TBS; pH 7.5)

   20 mM tris-base
   500 mM sodium chloride
   Distilled water

2) TTBS

   TBS
   0.05% Tween-20

3) Blocking solution

   10% skimmed milk powder in TTBS

4) Antibody diluent

   5% skimmed milk powder in TTBS

Primary antibody: Rabbit polyclonal antibody to human GLP-1, GIP, GHS and GPR39 receptors

Secondary antibody: sheep anti-rabbit IgG-HRP (horseradish peroxide)

ECL Western blotting detection reagents

Hyperfilm ECL

Developer

Fixer

2.11.4.2 Method

The membrane was removed from the blotting unit, placed in a container with 10 ml of blocking solution and incubated overnight at 4°C. The primary antibody solution was prepared by diluting 8 µl of primary antibody in 2 ml of antibody diluent for a

final dilution of 1:250 or 1:500. The membrane was placed in a clean bottle container (with the protein coated surface innermost) together with the primary antibody solution and placed on a rocker, rotating at 4°C overnight. The membrane was then transferred to a clean container and washed with 10 ml of TTBS by incubating on the rocker for 15 min at room temperature. This procedure was repeated twice more, using fresh TTBS on each occasion.

After these washing steps the membrane was incubated with 2 ml of secondary antibody solution (1:1000 dilution in antibody diluent), shaking for 1 h at room temperature. The membrane was washed 3 times in TTBS as before (5 min incubation only) followed by 3 further 5 min washes in TBS. The membrane was transferred to a clean container and incubated for 30 min in TBS, which was then poured off. For detection, 1 ml of each of the two ECL reagents was mixed, poured over the membrane and incubated for 1 min at room temperature. Excess reagent was then discarded and the membrane heat-sealed in Saran wrap.

The membrane was exposed within 5 min by placing it face up into an intensifying screen. A suitably sized piece of Hyperfilm ECL chemiluminescence film was placed on the membrane and intensifying screen shut. The exposure time was 5-10 min. the film was placed in developer for 2-3 min then transferred to fixer for at least 5 min. Finally, the film was rinsed in water for 10 min before being allowed to air dry.

2.12 Glucose uptake

Glucose transport is the process by which glucose is taken up into cells through a facilitated carrier process.

2.12.1 Materials

- In vitro differentiated adipocytes in 12-well plates (SGBS cells are optimal at day 10-12)
- KRH buffer (Krebs-Ringer-Hepes)

  130 mM NaCl

  10 mM HEPES

  10 mM $MgSO_4 x7H_2O$

  2.5 mM $NaH_2PO_4 xH_2O$

  4.6 mM KCl

2.5 mM $CaCl_2$ x$2H_2O$

- Adjust pH to 7.4
- Freshly add: 1% BSA
- ^{3}H-deoxyglucose – Final concentration in 4.5 cm^2 well (12 well) should be 0.2 µCi
- PBS
- Serum free basal medium
- Insulin
- 0.1 N NaOH

2.12.2 Working solution/ per well

- KRH/BSA 47.8 µl
- 30mM 2-deoxy-D-glucose 2.0 µl
- [3H]2-DG 0.2 µl

2.12.3 Method

A modification of a method by Bernier *et al.* (1988) was used for the measurements of 2-deoxy-D-glucose uptake. Briefly, SGBS fibroblasts were grown and differentiated in 12-well culture plates as described in section 2.4. After a 24 h incubation with pretreatment medium in the presence or absence of various concentrations of GLP-1, GIP, ghrelin or obestatin treatments, cells were washed twice and incubated with 950 µl of KRH buffer containing 1% BSA for 3 h at 37 °C, 5% CO_2. This was followed by the addition of different concentrations of insulin for 1 h at 37 °C (no insulin added for the basal uptake). 2-DG was added to a concentration of 60 µM containing 0.2 µCi/well of 2-deoxy-D- [1-^{3}H] glucose (Sp. Act 315 GBq/mmol, GE Healthcare) for 5 min at 37°C, 5% CO_2. Uptake was stopped by the addition of 2 ml of ice-cold PBS containing 200 µM phloretin (Sigma). The cells were washed three times with PBS stop solution, and solubilised in 0.1 N NaOH for 10 min at 22 °C. The cell lysates were subjected to liquid scintillation counting using EcoScint A fluid (National Diagnostics). The uptake of 2-DG was measured in the absence and presence of 40 µM cytochalasin B to correct for non-specific uptake.

2.13 Electrospray ionization mass spectrometry (ESI-MS)

The technique is called Electrospray Ionisation Mass Spectrometry (ESI-MS). As its name implies, it measures the mass (molecular weight) of molecules, by producing singly or multiply charged molecular ions from an analyte solution by spraying it (the solution) under the influence of a strong electrical field. The mass spectrometer used for this work is called a tandem mass spectrometer. It consists essentially of an ion source followed by two mass analysers in series with a collision cell in between them. In general the stages within the mass spectrometer are:

- Production of ions from the sample
- Separation of ions with different masses
- Detection of the number of ions of each mass produced
- Collection of data to generate the mass spectrum

A mass spectrometer measures the mass-to-charge ratio ***(m/z)*** of each ion peak by the following equation: $(m/z) = (M_r + nH) / n$

Where M_r molecular masses, H = proton mass = 1.00794 Da and n = number of protons (charges) for any particular ion peak.

2.13.1 Materials

- 20 µl stock solution:
  Obestatin amide solution (dissolved in DMSO)
  De-ionised water

- 180 µl denaturing solution which contains:
  De-ionised water
  50 % aqueous acetonitrile
  0.2 % formic acid

2.13.2 Method

The mass spectrometry study was kindly carried out by Mr Brian Green (VG BioTech/Fisons, Altrincham, England) using VG BioQ mass spectometer with electrospray (ES) ion source.

Initially, the obestatin amide sample is diluted 10-fold with water to give a stock solution. An aliquot of the stock solution made up into aqueous acetonitrile solutions (50:50 v/v)/ 2% formic acid, of final concentration 25-50 pmol/ µl. Generally, a 10 µl aliquot of the analyte solution is injected, via a loop injector, into a stream of the same solvent mixture flowing at a rate of 5 µl /min. The mass spectrometer is then routinely scanned over an appropriate m/z range, determined experimentally, generally several scans ≤15, are summed to obtain the final ESI-MS spectrum. Calibration of the *m/z* scale of the spectrometer is normally performed during each series of measurements using a solution of pure protein of known structure.

2.14 Statistics

The statistical significance of the difference between the means of two groups of samples was assessed by Student's *t*-test using Microsoft Excel 2003. The statistical differences between more than two groups (treatments vs control) however were evaluated through one-way ANOVA (Analysis of Variance) with Dunnett's post-hoc test using the GraphPad InStat version 3.00 for Windows 95{GraphPad Software, San Diego California USA}. Normality of the variables was tested and confirmed with the Kolmogorov and Smirnov method. Differences were considered to be significant when the P value was less than 0.05 ($P<0.05$). All results are presented as mean ± standard error of the mean (SE).

CHAPTER 3

GUT HORMONE RECEPTOR EXPRESSION IN MOUSE AND HUMAN ADIPOSE TISSUE

3.1 Introduction

With obesity on the rise worldwide, studies have focused on investigation of the whole body mechanisms that govern weight gain and the abnormalities of both metabolic and inflammatory pathways. However, a potential role for gut peptides in white adipose function has been little considered and much of the current research on gut peptides is focused on circulating gut peptide levels but little work has been directed at studying receptors for gut peptides.

In general, the receptors for the peptide hormones are located in the plasma membrane. Receptor structure is varied: some receptors consist of a single polypeptide chain with a domain on either side of the membrane, connected by a membrane-spanning domain. Some receptors are comprised of a single polypeptide chain that is passed back and forth in serpentine fashion across the membrane, giving multiple intracellular, transmembrane, and extracellular domains. Other receptors are composed of multiple polypeptides. For example, the insulin receptor is a disulfide-linked tetramer with the β-subunits spanning the membrane and the α-subunits located on the exterior surface. The hormone-binding signal of most, but not all, plasma membrane receptors is transduced to the interior of cells by the binding of receptor-ligand complexes to a series of membrane-localized GDP/GTP binding proteins known as G-proteins.

The gastrointestinal tract is the source of five named hormones and a large number of peptides whose range of functions and target tissues remain to be identified (Rehfeld 1998; Holst *et al.,* 1996). Gut peptides and hormones have effects in multiple tissues (Sancho *et al.,* 2006; Yip *et al.,* 2000). These peptides stimulate whole body metabolism through both direct and indirect pathways. Generally, the effects of the incretins, GLP-1 and GIP, in stimulating insulin secretion in response to an oral glucose load (Sancho *et al.,* 2006), are mediated following activation of the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR) respectively. Both GLP-1R and GIPR are typical seven-transmembrane spanning receptors coupled to G-protein activation. GLP-1R increased cAMP production and activation of PKA. However, there are also PKA-independent responses initiated through the GLP-1R (Miki *et al.*, 1996).

Moreover, studies reported that GIPR has alternative modes of action, both dependent and independent of cAMP, in rat adipocytes (Ehses *et al.,* 2002) and in non-adipose cell lines (Oben *et al.,* 1991a; Beck *et al.,* 1983; Beck *et al.,* 1988).

The two newly discovered gastric mucosa-derived peptides, ghrelin and obestatin, appear to have opposing roles in the control of appetite (Zhang *et al.,* 2005). Although both peptides derive from a common pre-prohormone, ghrelin is orexigenic whereas obestatin is anorexigenic. Differential post-translation events determine whether the signal to eat or not to eat predominates. Furthermore, ghrelin and obestatin peptides are activated by different receptors. The receptor for ghrelin is growth hormone secretagogue receptor (GHS-R) (Van der Lely *et al.,* 2006) and the proposed receptor for obestatin is one of the orphan receptors, G-protein coupled receptor 39 (GPR39) (Zhang *et al.,* 2005). It should be noted, however, that recent data question whether or not GRP-39 is, in fact, the endogenous receptor for obestatin (Holst *et al.,* 2007; Lauwers *et al.,* 2006). Therefore, the role of gut peptides in whole body metabolism extends beyond regulation of only gastrointestinal function. These peptides, with receptors located throughout the body, may play a more active role in whole body metabolism, appetite regulation and fuel preference than is currently appreciated.

Elucidation of the roles of gastrointestinal hormones in adipose tissue function may lead to further understanding of the mechanisms of obesity which in turn could allow more effective approaches to the prevention and treatment of obesity. Thus, if specific receptors for gut peptides can be found in adipose tissue one may infer that it is likely that they may have a direct effect on adipose tissue function. While specific receptors for GIP have been identified, studies have been inconclusive with regard to GLP-1 and very little is known regarding ghrelin and obestatin.

Therefore, as a starting point in an examination of potential additional physiological actions of gut hormones in obesity, the expression of receptors from the gut were examined for the incretins, GIPR and GLP-1R, the orexigenic peptide ghrelin, GHS-R, and the anorexigenic peptide obestatin, GRP39, in nonadipose and adipose mouse tissue, in two major human fat pad (subcutaneous and omental) and in cell culture systems of both mouse and human.

3.2 Methods

3.2.1 Mouse tissue

Adult male CD-1 mice, aged 8 wk, were obtained from Harlan Olac (Bicester, UK). The mice were housed on receipt for 2 wk at 21°C with a 12:12-h light-dark cycle (lights on at 0700) and fed a commercial rodent diet (CRM Diet, Labsure, Witham, UK) containing 19.2% protein and 4.3% lipid (wt/wt). Both food and water were available *ad libitum*. The mice were killed by cervical dislocation, and the following tissues were rapidly removed and frozen in liquid nitrogen: skeletal muscle, small intestine, liver, kidney, brain and various white fat depots (gonadal, mesenteric, omental, and subcutaneous). The omental depot, which is small and can be hard to localize in lean mice, is located alongside the inferior surface of the stomach and is distinct from the mesenteric fat. All tissues were stored at -80°C until analysis.

3.2.2 Human WAT

Abdominal subcutaneous and omental adipose tissue were provided by Dr S Wong (University of Liverpool) following gastroplasty of obese patients (aged 35-48 years, BMI>43 kg/m^2); the subjects did not exhibit any other ongoing diseases. After removal, all tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

cDNA samples from human subcutaneous depots derived from 4 obese subjects (age, 50 ± 16 yr; BMI 45 ±5 kg/m^2) and 4 lean or nearly normal weight controls (age, 50 ±11 yr; BMI 24 ± 2.5 kg/m^2) were kindly provided by Dr M Bulló (Universitat Rovira I Virgili, Reus, Spain). The tissue was obtained by biopsies or during surgical procedures (cholecystectomy or abdominal hernia repair).

3.2.3 3T3-L1 cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, USA) and cultured at 37°C in a humidified atmosphere of 5% CO_2/ 95% air as

described in section 2.3.1. The cells were maintained in culture medium containing Dulbecco's modified Eagle's medium (DMEM), and 10% foetal calf serum (FCS). Differentiation of the cells was initiated 24 h after confluence by incubation for 2 days in culture medium containing dexamethasone, IBMX and insulin. This was followed by maintenance in feeding medium (renewed every 2 or 3 days) consisting of culture medium containing insulin. To investigate the time course of receptor gene expression during adipocyte differentiation, cells were collected every 2 days for up to 15 days.

3.2.4 SGBS cell culture

SGBS preadipocytes were kindly supplied by Professor Martin Wabitsch (University of Ulm, Germany). SGBS adipocytes were cultured as described in section 2.4.2. The preadipocytes were maintained in culture medium until they were totally confluent. Differentiation of the cells was initiated 24 h after post-confluence by exposing the cells to the adipogenesis cocktail for 4 days. After this step, the cells were maintained in feeding medium (containing insulin, cortisol and T_3) for up to 18 days and collected every 1-3 day for a time course study. The accumulation of lipid droplets in the differentiated SGBS cells was confirmed by viewing under a light microscope. More than 90% of preadipocytes differentiated into mature fat cells.

3.2.5 RT -PCR

RNA isolation was as described in section 2.6.1. The concentration of RNA was measured by spectrophotometry (Section 2.5.3). One μg of RNA was reverse transcribed to cDNA (Section 2.6.2) and stored at -20°C until being used for PCR (Section 2.7).

3.2.5 Western blotting

Protein extraction was performed using the methods described in section 2.11.1. The total protein was quantified using the BCA assay described in section 2.11.2. Samples containing 10-20 μg of total proteins were separated by 12% SDS-PAGE (Section 2.11.3).

3.3 Results

3.3.1 Gut peptide receptor gene expression in mouse tissue

3.3.1.1 Incretin receptor gene expression in mouse tissue (adipose and nonadipose)

In initial studies, the presence of the mRNAs for GIPR and GLP-1R mRNA in the major fat depots and various nonadipose tissues of mice was examined by RT-PCR. A strong signal for GIPR mRNA was obtained in each of the four white adipose tissue sites examined (subcutaneous, gonadal, omental and mesenteric) and in the brain (Fig 3.1and Fig 3.2). A faint signal was discernible with the small intestine, but no signal was detected in skeletal muscle, liver or kidney (Fig 3.1A). By contrast, amplification of GLP-1R was widely detected in the entire nonadipose mouse tissues studied (skeletal muscle, small intestine, liver, kidney and brain) (Fig 3.1A). On the other hand, GLP-1R mRNA expression was found only in omental and mesenteric fat depots but there was no detectable GLP-1 receptor gene expression in subcutaneous or gonadal fat (Fig 3.2). Sequence analysis of the PCR products confirmed the identity. Studies were limited with regard to GLP-1 receptor expression in the liver. The results from this section supported the outcome of a previous study (Schmidtler *et al.,* 1994), in which the mRNA of GLP-1R was detected in the rat liver using a combination of northern blot analysis and RT-PCR. For the subsequent experiment, a cDNA sample of human liver tissue was kindly provided by Dr. Stuart Wood (University of Liverpool). Importantly, this study showed that GLP-1R gene expression was detectable in both mouse and human liver (Fig 3.1B).

3.3.1.2 GHS-R and GPR39 gene expression in mouse tissue (adipose and nonadipose)

The presence of GHS-R and GPR39 mRNA in four major WAT depots (subcutaneous, gonadal, omental and mesenteric) and also in skeletal muscle, small intestine, liver, kidney and brain of mouse was examined by RT-PCR.

There was widespread tissue expression of the GPR39 gene; GPR39 mRNA was present in all the adipose and nonadipose tissue examined (Fig 3.1 and Fig 3.2). In

contrast, GHS-R gene expression was detected only in three major fat depots (subcutaneous, gonadal and omental) and in the brain of the mouse tissues (Fig 3.1 and Fig 3.2). GHS-R mRNA was not detected in mesenteric fat, skeletal muscle, small intestine, liver or kidney by RT-PCR.

3.3.2 Gut peptide receptor gene and protein expression in human white adipose tissue

3.3.2.1 Incretin receptor gene and protein expression in human WAT

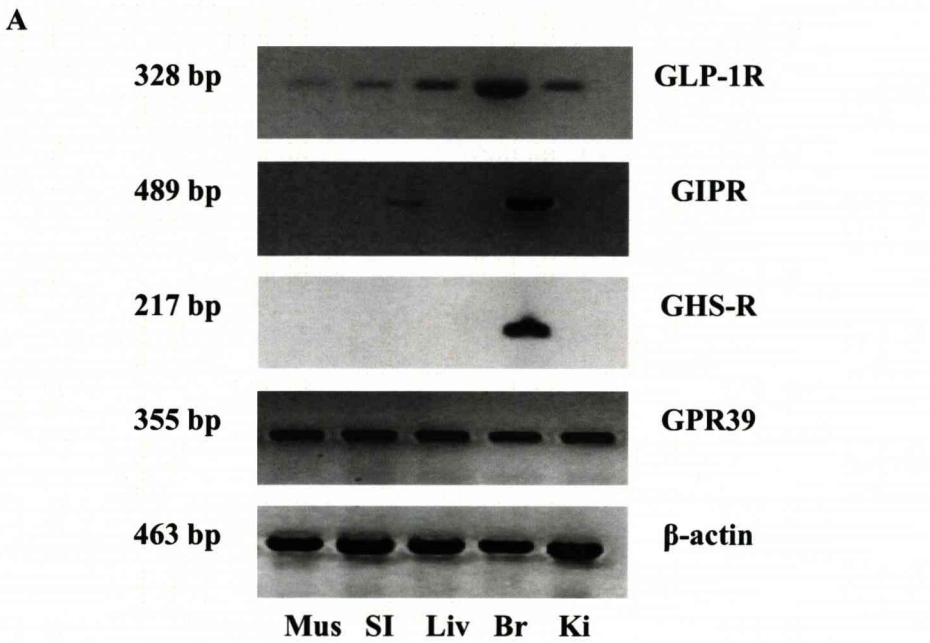
In the subsequent study, RT-PCR and western blotting were employed to examine whether the gene encoding incretin receptors are expressed in human WAT and whether the receptor protein is present. Expression of the genes encoding both GLP-1R and GIPR was examined in the major human WAT sites. Signals for GIPR mRNA were observed in both human depots examined, namely subcutaneous and omental fat; the signal was relatively strong in both WAT depots investigated (Fig 3.3A). A signal for GLP-1R mRNA was only observed in the omental fat depot; no signal for GLP-1R mRNA was detected in the subcutaneous tissue.

To investigate whether the mRNA coding sequence of GIPR and GLP-1R are translated into a functional protein, the expression of receptor proteins, both in human subcutaneous and omental fat pads, of two subjects was assessed by western blotting using specific polyclonal antiGIPR and antiGLP-1R antibodies respectively. Immunoreactivity was obtained with a band of a 53 kDa in both fat depots, consistent with the presence of GIPR. In contrast, GLP-1R protein expression was detected only in the omental fat depot of both human subjects, and was not detected in subcutaneous fat (Fig 3.3B).

3.3.2.2 GHS-R and GPR39 gene and protein expression in human WAT

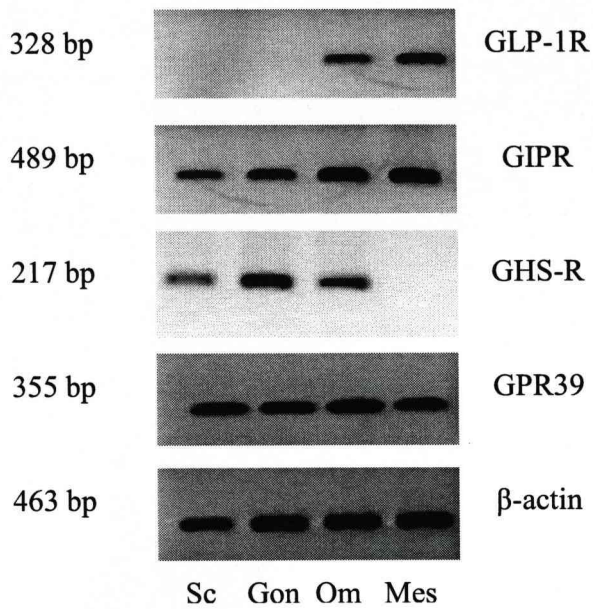
The studies on GHS-R and GPR39 gene expression reported in the previous section (3.3.1.2) involved a range of nonadipose and four major adipose mouse tissues. RT-PCR was used to investigate whether the genes are also expressed in human WAT. Figure 3.4A shows that GHS-R and GPR39 mRNA was present in both human depots

Figure 3.1 GLP-1R, GIP R, GHS-R and GPR39 expression in various nonadipose mouse tissues



(A) GLP-1R, GIPR, GHS-R and GPR39 gene expression using RT-PCR, in several nonadipose mouse tissues. (B) GLP-1 receptor gene expression in both mouse and human liver. Mus, skeletal muscle; SI, small intestine; Liv, liver; Br, brain; Ki, kidney. Cycle number for GLP-1R, GIPR, GHS-R, GPR39 and ß-actin are 36, 32, 36, 32 and 25 cycles respectively

Figure 3.2 GLP-1R, GIP R, GHS-R and GPR39 expression in major mouse fat depots



GLP-1R, GIPR, GHS-R and GPR39 mRNA is detected in four major mouse WAT depots using RT-PCR. Sc, subcutaneous; Gon, Gonadal; Om, omental; Mes, mesenteric. Cycle number for GLP-1R, GIPR, GHS-R, GPR39 and ß-actin are 36, 32, 36, 32 and 25 cycles respectively.

studied, subcutaneous and omental, the signal was rather stronger, however, for the GPR39 gene compared to the GHS-R gene (Fig 3.3A).

In the subsequent studies, proteins were extracted from major human WAT depots, precisely subcutaneous and omental fat of two human subjects to explore GHS-R and GPR39 protein expression in human WAT by western blotting. Signals for GHS-R and GPR39 proteins were detected in both omental and subcutaneous fat depot of the two subjects, which paralleled the observation of GHS-R and GPR39 gene expression in these major human fat depots (Fig 3.3B).

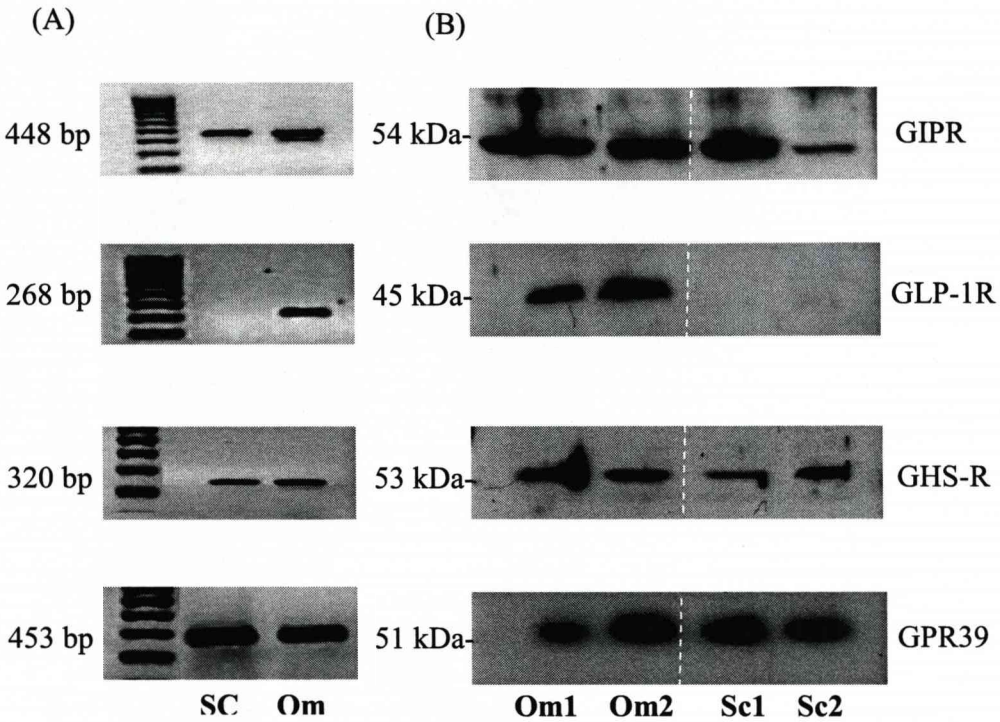
3.3.3 Gut peptide receptor gene and protein expression in cell culture system

3.3.3.1 Incretin receptor gene expression in mouse 3T3-L1 cells

In the following experiments, cultured mouse 3T3-L1 cells were employed to investigate the expression of both GIPR and GLP-1R mRNA in adipocytes pre- and post- induction of differentiation using RT-PCR.

GIPR was not detected in undifferentiated 3T3- L1 cells, but was evident by day 5 after the induction of differentiation into adipocytes and the signal remained high over the following 10 days (Fig 3.4). A similar pattern was evident with GLP-1R, but this gene was expressed even later; mRNA was detected from day 8 post-differentiation and onwards (Fig 3.4).

Figure 3.3 GLP-1R, GIP R, GHS-R and GPR39 gene and protein expression in major human fat depots



(A) GIPR, GLP-1R, GHS-R and GPR39 gene expression using RT-PCR in subcutaneous and omental WAT depots. Cycle number for GLP-1R, GIPR, GHS-R and GPR39 are 36, 34, 36 and 32 cycles respectively (B) Receptor protein examined by western blotting in two major human WAT depots (subcutaneous and omental) of two subjects (1-2). Sc, subcutaneous; Om, omental.

3.3.3.2 GHS-R and GPR39 gene expression in mouse 3T3-L1 cells

In the next studies, 3T3-L1 cells were utilised before and after the induction of differentiation into adipocytes to assess whether GHS-R and GPR39 mRNAs are present by RT-PCR. GPR39 mRNA was undetectable in 3T3-L1 preadipocytes; however, a signal was immediately observed at day 1 post-induction and was present throughout the subsequent time course after the initiation of differentiation (Fig 3.4). In contrast to GPR39 mRNA, GHS-R mRNA was not detected before or shortly after the induction of differentiation; a signal was, however, present at day 8 post induction and thereafter (Fig 3.4).

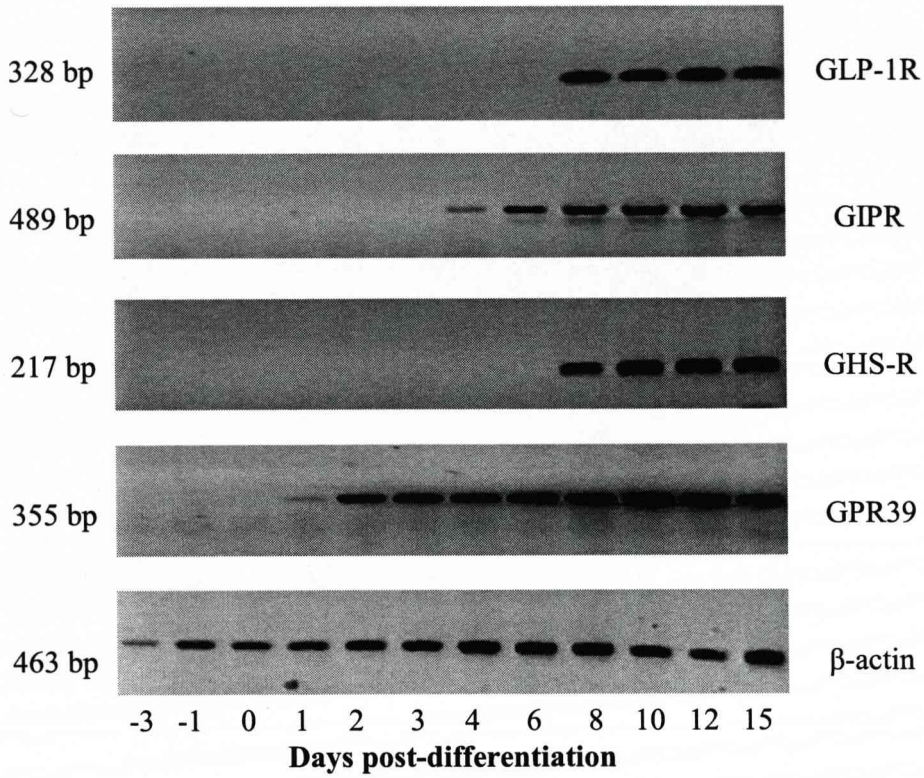
3.3.3.3 Incretin receptor gene and protein expression in human SGBS adipocytes

To further establish whether the incretin receptor genes are expressed in differentiated adipocytes, another adipocyte culture system (SGBS) was employed. SGBS cells are a human preadipocyte cell strain derived from the stromal-vascular fraction of the subcutaneous white adipose tissue of an infant with Simpson-Golabi-Behmel syndrome. The cell strain shows a high capacity for adipose differentiation, resulting in mature fat cells which are biochemically and functionally similar to human adipocytes (Wabitsch *et al.,* 2001).

This study showed that GIPR gene expression was undetectable in preadipocytes; in contrast, a strong signal for GIPR mRNA was detected at day 4 and thereafter (Fig 3.5). A similar pattern was evident with GLP-1R, but was expressed even later; mRNA was detected by day 8 post-differentiation and thereafter (Fig 3.5).

In the subsequent studies, the total protein was extracted from pre- and post-differentiated SGBS cells to explore the GLP-1R and GIPR protein expression in human adipocytes by western blotting. Signals from the GIPR protein began to be detected at day 4 and thereafter (Fig. 3.6). In contrast, GLP-1R protein signals were very faint, and were detected very late (day 8) after the induction of differentiation and onwards (Fig. 3.6). However, no signal for both incretin receptor proteins was identified in SGBS preadipocytes, which paralleled the observation of the receptor

Figure 3.4 Receptor expression during the differentiation and development of 3T3L1 cells to adipocytes



GIP R, GLP-1R, GHS-R and GPR39 gene expression in cultured 3T3-L1 cells pre- and post- differentiation assessed by RT-PCR. Differentiation was initiated on day 0. Cycle number for GLP-1R, GIPR, GHS-R, GPR39 and ß-actin are 36, 32, 36, 32 and 25 cycles respectively.

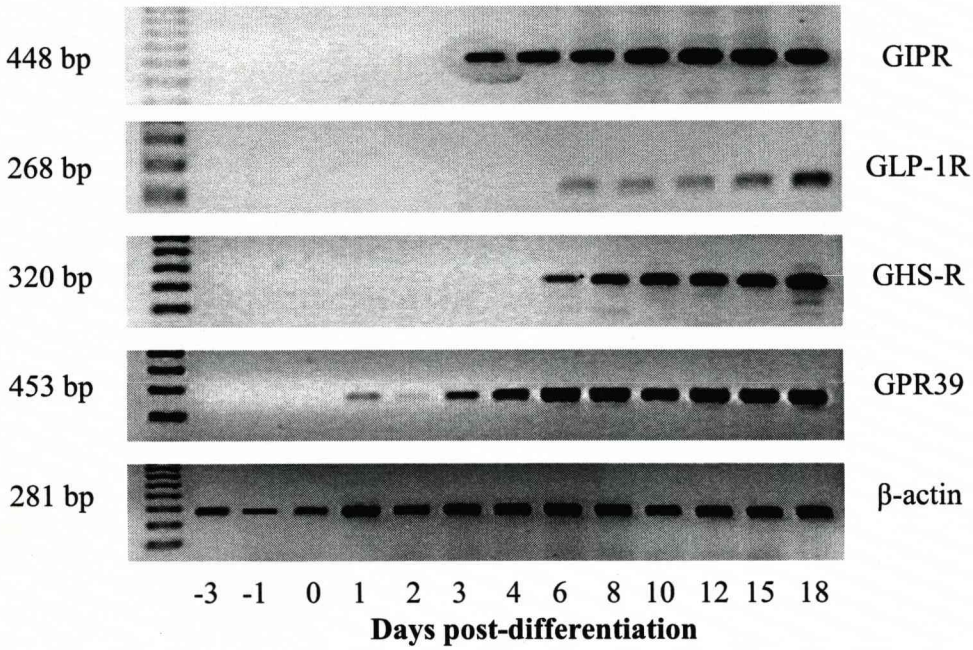
gene expression in the cells (Fig 3.5).

3.3.3.4 GHS-R and GPR39 gene and protein expression in human SGBS adipocytes

GPR39 mRNA was undetectable in SGBS preadipocytes; however, a signal was immediately observed at day 1 post-induction and was present throughout the subsequent time course (Fig 3.5). GHS-R mRNA appeared much later, and signal was not detected in undifferentiated SGBS cells, but was evident by day 6 after the induction of differentiation into adipocytes (Fig 3.5).

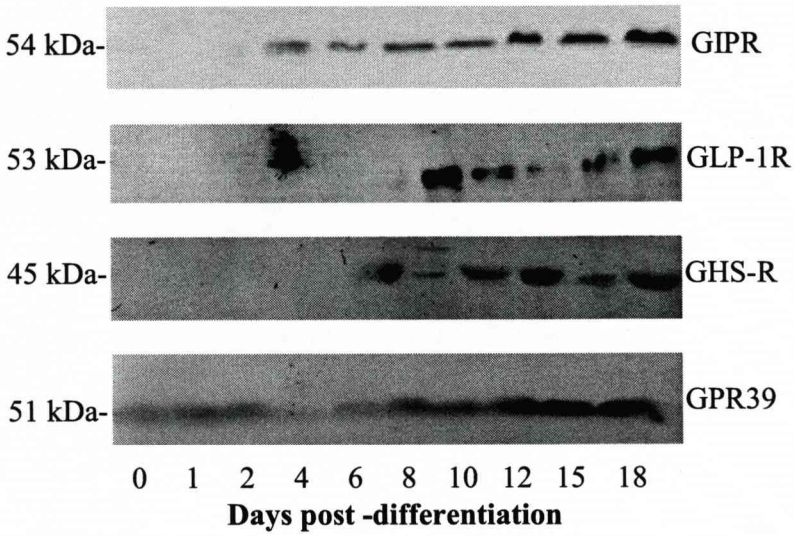
To investigate the expression of GPR39 and GHS-R protein, both in pre- and post--differentiated SGBS cells, western blotting was employed using a specific polyclonal antiGPR39 and antiGHS-R antibodies, respectively. GPR39 protein was detectable in preadipocytes; a faint signal was immediately observed at day 0 and became stronger at day 2 post-differentiation and moreover was present throughout the subsequent time course (Fig 3.6). In contrast to GPR39, GHS-R protein was not detected before or shortly after the induction of differentiation; a signal was, however, present at day 6 post induction and thereafter (Fig 3.6).

Figure 3.5 Receptor gene expression in human SGBS adipocytes



GIP R, GLP-1R, GHS-R and GPR39 gene expression in cultured SGBS cells pre- and post- differentiation assessed by RT-PCR. Differentiation was initiated on day 0. Cycle number for GLP-1R, GIPR, GHS-R, GPR39 and ß-actin are 36, 34, 36, 32 and 23 cycles respectively.

Figure 3.6 Receptor protein expression in human SGBS adipocytes



Western blotting was performed to detect GIPR, GLP-1R, GHS-R and GPR39 protein expression during differentiation and development of SGBS cells to adipocytes.

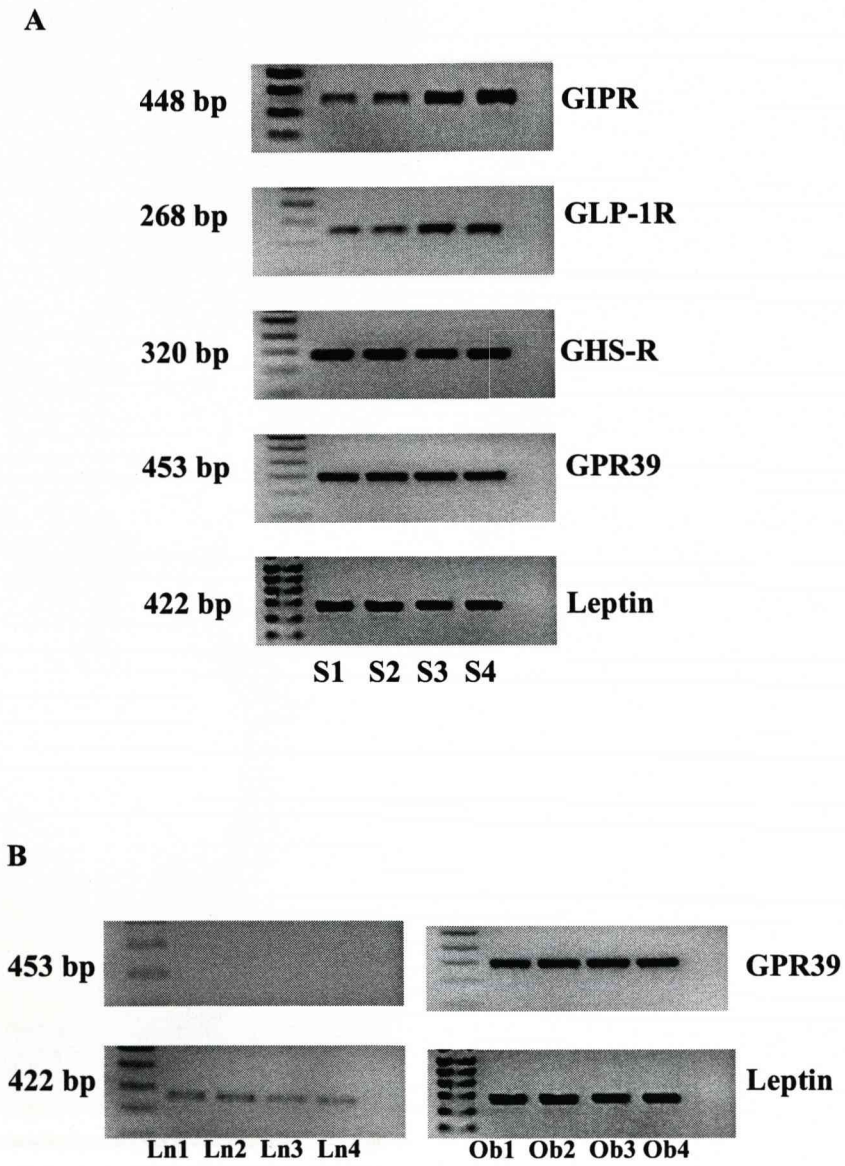
3.3.4 Gut peptide receptor gene expression in WAT of obese subjects

The next experiments examined whether the mRNA of genes encoding the receptors for ghrelin (GHS-R), obestatin (GPR39), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) are expressed in WAT of human obese subjects using RT- PCR.

This study was carried out by using the subcutaneous fat depots obtained from four morbidly obese individuals. Leptin mRNA was examined as a reference for the same obese subjects. Expression of all the above gut peptide receptor genes, together with that of the leptin gene, was detected in all four obese human (subcutaneous) WAT subjects (Fig 3.7A).

From the above observations, GPR39 and the other receptor genes expression are evidently expressed in obese human (subcutaneous) WAT. The mRNA of the GPR39 gene was then examined again in subcutaneous fat of four lean individuals using RT-PCR. Although, GPR39 gene expression was found in subcutaneous fat of all four human obese subjects, no expression was evident for GPR39 in the lean subjects (Fig 3.7B). For a reference, the mRNA levels of leptin in the same samples of lean and obese subjects were also examined using RT- PCR (Fig 3.7B).

Figure 3.7 Incretins R, GHS-R and GPR39 expression in subcutaneous WAT depot of obese human subjects



(A) GIPR, GLP-1R, GHS-R and GPR39 gene expression using RT-PCR, in subcutaneous WAT of four (S1-S4) obese human subjects. Leptin expression was examined as a reference (B) Leptin and GPR39 were examined in subcutaneous fat of four human obese (Ob1-4) and lean (Ln1-4) subjects. Cycle numbers for GLP-1R, GIPR, GHS-R, GPR39 and Leptin are 36, 34, 36, 32 and 33 cycles respectively.

3.4 Discussion

The literature review indicates that whether the gut hormone receptor(s) gene examined in this study including, the incretins GIP and GLP-1, ghrelin and obestatin, are expressed in white adipose tissue has been an issue of debate. The initial experiments set out to establish whether the genes encoding the receptors for the above gut peptides are indeed expressed in adipocytes. RT-PCR was performed on RNA extracted from several adipose and nonadipose tissues, including four major WAT depots (subcutaneous, gonadal, omental and mesenteric) in mice. In addition, subcutaneous and omental WAT samples of lean and obese human subjects, and adipocytes from cell culture systems (mouse 3T3-L1 and human SGBS cells) were examined. The receptor protein was determined in adipose tissues by using western blotting.

3.4.1 Gut peptide receptor gene expression in mouse tissue

In the present study, GLP-1R, GIPR, GHS-R and GPR39 gene expression was detected in the mouse brain, consistent with the observations of previous studies in rodent brain (Wei & Mojsov,1995; McKee *et al.,* 1997; Rudovich *et al.,* 2007). The presence of binding sites in the brain suggest that the above gut peptide receptor(s) may have a role as neurotransmitters. However the action of GIP and the more recently discovered peptide, obestatin, in the central nervous system remain uncertain.

Using RT- PCR the presence of GLP-1R and GPR39 mRNA has been shown in a range of mouse tissue including skeletal muscle, small intestine, liver, kidney and brain. The tissue expression pattern of both GLP-1R and GPR39 indicates that these receptors might be truly essential for the function of a number of metabolic organs, although this result was not quantitative.

Importantly, GLP-1R gene expression was detected not only in mouse liver, but also in human liver tissue, which supports the outcome of other studies (Schmidtler *et al.,* 1994; Villanueva-Peñacarrillo *et al.,* 1995), in which the mRNA expression of GLP-1R was detected in mouse and rat liver using a northern blot analysis and RT-PCR

respectively. The known insulin secretion-independent effect of GLP-1 on hepatic glucose uptake is consistent with the presence of specific GLP-1 receptors on liver cells. Binding of GLP-1 to these receptors could initiate intracellular signaling pathways which could target kinases and/or factors involved in glucose uptake and glycogen synthesis. Recent evidence suggests that GLP-1 is secreted into the hepatic portal vein through the presence of GLP-1 sensors or receptors in the hepato-portal region (Burcelin *et al.,* 2001). Because of the rapid degradation of GLP-1 in the plasma, the hepato-portal region may play a critical role in the generation of the full effects of GLP-1.

3.4.2 Gut peptide receptor gene expression in mouse and human WAT

The present study has identified receptors for multiple gut peptides in major white adipose tissue depots of both mice and humans. Whether any of these receptors have a normal physiological role in the control of fat metabolism remains to be determined. Differences in GLP-1R gene expression between subcutaneous and visceral fat have been shown and this may explain why previous reports on the lipolytic or lipogenic activity of incretins in rodent adipocytes have been contradictory; some appear to support the notion of increased lipogenesis, while some oppose it.

GLP-1R gene expression was found in omental and mesenteric, but not subcutaneous or gonadal WAT of mice. It is tempting to speculate about the significance of the absence of GLP-1R mRNA in subcutaneous fat while being abundant in omental fat in humans. As discussed in Chapter 1, WAT contains mature adipocytes and the stromal-vascular (SV) fraction, which comprises macrophages, fibroblasts, vascular endothelial cells and preadipocytes (Hausman, 1985). The differences in GLP-1 gene expression could come from any component of these fat depots. Therefore, additional study is required to establish the cellular site not only of GLP-1R, but also of GIPR, GHS-R and GPR-39 gene expression in adipose tissue.

Adipose tissue located within the abdominal cavity has been suggested to be functionally and metabolically distinct from that of the subcutaneous compartment and these differences could play a role in obesity-related complications (Vohl *et al.,* 2004). The findings presented here, however, could be the result of a different

regulatory mechanism of subcutaneous and visceral fat gene expression by multi-stimuli. Vohl *et al.* (2004), showed by using multiple array replicates, that the expression profiles of different genes involved in lipolytic stimuli and cytokine secretion, were altered in subcutaneous and visceral adipose tissues of obese men. Moreover, Tchkonia *et al.,* (2006), demonstrated regional differences in the intrinsic characteristics of the preadipocytes in the different fat depots, with those of subcutaneous adipose tissue presenting greater differentiation and fat cell gene expression but less apoptosis than that of visceral fat tissue.

The regulation of GLP-1R expression in human fat tissue has until now not been investigated in detail. In recent years, it has been shown that the promoter region of the incretin receptor genes contains multiple Sp1/Sp3 binding sites, which appear to be involved in the cellular expression of the receptor (Barth *et al.,* 2002). However, Sp1 binding activity is enhanced during adipocyte differentiation and acts predominantly as an activator of G-protein coupled receptor activity, while Sp3 may also exert a repressor activity (Barth *et al.,* 2002). Thus, fat depot-specific differences in GLP-1R gene expression may be explained via the depot-specific composition of slowly and rapidly replicating subtypes of preadipocytes (Tchkonia *et al.,* 2005).

3.4.3 Gut peptide receptor gene and protein expression in adipocytes in cell culture

By using 3T3-L1 cells in culture, the current study further indicates, that not only the incretin receptor genes but also the GHS-R and GPR39 genes are expressed after differentiation, although not in preadipocytes. The signals for the GIP, GLP-1 and ghrelin receptor mRNAs are late post differentiation, emerging from day 4, day 8 and day 8, respectively. However, a signal for the GPR39 gene was immediately observed at day 1 post-induction and was present throughout the subsequent time course shortly after adipocyte differentiation. This finding is partially consistent with previous reports suggesting that GIPR, GLP-1R and GHS-R gene expression is detected in 3T3-L1cells at an even earlier stage (McIntosh *et al.,* 1999; Egan *et al.*, 1994; Kim *et al.,* 2004). On the other hand, these are the first data which demonstrate that adipocyte cell culture systems of mice 3T3-L1 cells express the transcript coding for the proposed obestatin receptor (GPR39).

The expression of gut peptide receptor genes in differentiated mouse adipocytes, but not in preadipocytes, was further supported by exploring GIPR, GLP-1R, GHS-R and GPR39 gene and protein expression in human SGBS adipocytes. GPR39 mRNA was undetectable in SGBS preadipocytes; however, a signal was observed at day 1 post-induction and was present throughout the subsequent time course. In contrast to GPR39, GIPR, GLP-1R and GHS-R mRNA appeared much later. The early presence of GPR39 mRNA and protein during the development process of the adipocytes implies that GPR39 may be an early marker of the differentiation in SGBS adipocytes.

The presence of these gut peptide receptors in fat cells could be an indicative of the physiological nature of the effects mediated by these gut peptides in adipose tissue. To date, surprisingly little is known about these gut peptides interactions with adipocyte biology. However, Tnag *et al.*, (2008), have recently evaluated the effect of obestatin on cell proliferation in primary cultures of piglet adipose cells. The results showed that this peptide induced cell proliferation in a dose-dependent manner with MEK/ERK 1/2 phosphorylation (Tnag *et al.,* 2008). However, in another study obestatin did not increase the cell cycle or viability of the murine 3T3-L1 adipocyte cell line, other than that this peptide inhibited the proliferation and differentiation of 3T3-L1 preadipocytes. Furthermore, the effect of obestatin was contrary to that of ghrelin on this adipocyte cell line (Zhang *et al.,* 2007). These findings suggest that direct effects of obestatin on proliferation and or differentiation, in adipocytes may play a role in regulating body fat mass.

On the other hand, GLP-1 seems to exert differential, concentration-dependent effects on lipid metabolism in human adipocytes, which may be explained by the existence of different GLP-1-binding receptors (Villanueva-Penacarrillo *et al.*, 2001). Furthermore, recent studies showed that ghrelin activates the mitogen-activated protein kinase pathway *in vitro,* which stimulates cellular proliferation and differentiation in cultured white adipocytes. (Kim *et al.*, 2004; Zhang *et al.*, 2004).

3.4.4 Gut peptide receptor gene expression in WAT of obese subjects

Finally, in this section of the study, receptor mRNA was detected in subcutaneous WAT of four obese human subjects. In addition to expression of GIPR, GLP-1R and GHS-R genes, GPR39 was evidently present in all obese human WAT used.

The GLP-1R gene expression in subcutaneous fat of the 4 obese subjects was not similar to what was revealed in the previous section of this chapter in lean subcutaneous fat. It has been postulated that an exaggerated 'incretin' factor, that induces glucose-dependent insulin secretion from the pancreas when the meal is taken orally, may have a pathophysiological role in obesity. Moreover the hyper- secretion of GLP-1 may be the long sought 'incretin' factor in obesity. Molavi *et al.*, (2006), suggested that obesity often produces a type 2-like diabetic state, which resolves after weight loss (Molavi *et al.,* 2006). That might explain the results in the current experiment in which the BMI of the 4 obese subjects used was 45 ±5 kg/m^2. Since this is classified as morbidly obese, we can therefore hypothesise that the obese subjects might have either an expanded or contracted end organ receptor number for this peptide.

Importantly, GPR39 gene expression was found in the subcutaneous fat of all four human obese subjects, but no expression was evident in lean subjects. It could be hypothesised that high expression of the GPR39 gene in obese subjects is due to a decreased obestatin level in obesity. In a recent study, Huda *et al.*, (2008) have shown that plasma obestatin concentrations are significantly lower in obese subjects when compared to lean controls. Nevertheless, the reduction of this peptide in obese humans is probably maladaptive and may further propagate the obese state. In another study, Catalan *et al.*, (2007), revealed that the GPR39 receptor is present in adipose tissue and that it is down regulated in obese type 2 diabetes, but not in non-diabetic obese subjects.

The work described in this chapter now shows that genes encoding the receptors for ghrelin (GHS-R), obestatin (GPR39), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) are expressed in the main WAT depots

of mice and humans, and are indeed expressed in differentiated cell culture systems of both mice and human adipocytes.

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CHAPTER 4

EFFECT OF GLP-1, GIP & GHRELIN ON ADIPOKINE GENE EXPRESSION IN HUMAN ADIPOCYTES

4.1 Introduction

In Chapter 3, it was demonstrated that the receptors, encoding the incretins, GLP-1 and GIP, and ghrelin, were expressed by human white adipose tissue, and that these gut receptors (mRNA and protein) were unambiguously detected in post differentiated 3T3-L1 and SGBS adipocytes. The finding of receptor expression in SGBS cells provides an opportunity to further study the possible biological roles of GLP-1, GIP and ghrelin peptides in human adipocytes. In this chapter, studies focused on exploring the possible regulatory effect of GLP-1, GIP and ghrelin on key adipokines in adipocytes. The studies were carried out by treating cultured human adipocytes with recombinant human GLP-1, GIP or ghrelin peptides in *vitro*. As described in the previous chapter, SGBS cells can maintain their proliferation and differentiation capacity after repeated subculture; more importantly, the differentiated adipocytes are histologically and metabolically similar to the adipocytes derived from the SV fraction in primary culture (Wabitsch *et al.,* 2001).

A limited number of investigations have demonstrated that gastrointestinal hormones may have an important role in the control of the pathways of lipid metabolism. For instance, GIP has been proposed to be an anabolic hormone for adipocyte lipid metabolism and stimulates lipoprotein lipase activity (Eckel *et al.,* 1979), thereby promoting fat storage. GIP also promotes fatty acid incorporation into adipose tissue (Beck *et al.,* 1983). GIP infusion promotes triglyceride clearance from the circulation in dogs (Wasada *et al.,* 1981), and inhibits the lipolytic effect of glucagon on adipocytes (Hauner *et al.,* 1988).

Unlike GIP, the effects of GLP-l on the lipolytic or lipogenic activity in rodent adipocytes have been contradictory, some supporting the notion of increased lipogenesis (Oben *et al.*, 1991; Egan *et al.*, 1994; Perea *et al.*, 1997), and some the opposite (Ruiz-Grande *et al.*, 1992).

Recently, ghrelin has been found to activate the mitogen-activated protein kinase pathway *in vitro* (Kim *et al.*, 2004; Zhang *et al.*, 2004), which stimulates cellular proliferation and differentiation in cultured white adipocytes (Kim *et al.*, 2004; Zhang *et al.*, 2004).

In this set of studies, synthetic GLP-1, GIP and recombinant ghrelin were used to investigate whether these peptides influence the expression of key adipokine genes in adipocytes. Little has been reported in regard to the regulation of adipokine production by incretins. However, a recent study has now examined the possibility that specific actions of GIP are mediated through the direct regulation of resistin release in 3T3-L1 adipocytes, resulting in increasing the activity of both PKB and LPL pathways (Kim *et al.,* 2007). On the other hand, ghrelin directly suppresses adiponectin mRNA expression (Ott *et al.*, 2002). In one study, intra-peritoneal injection of ghrelin increased both adiponectin and leptin expression in adipose tissue, but these effects did not reach statistical significance (Asakawa *et al.*, 2003).

In this section, the effect of GLP-1, GIP and ghrelin on the gene expression of leptin, adiponectin, MCP-1 and IL-6, was investigated in SGBS cells.

4.2 Methods

4.2.1 SGBS cell culture

SGBS cells were cultured as described in section 2.4.2. The preadipocytes were maintained in culture medium and differentiation of the cells was initiated 24 h post-confluence by incubating the cells for 4 days in induction medium. The cells were maintained in feeding medium, which was renewed 2-3 times a week. At day 12, the cells were pre-incubated with cortisol and T_3- free feeding medium for 24 h. All the human recombinant gut peptides explored in this set of studies were dissolved either in 100% DMSO or autoclaved water according to the protocol provided for each peptide, and the concentration of stock solutions for each peptide was 1 μM. Prior to treatment, incubation media were prepared by adding each peptide stock, to the cortisol and T_3-free feeding medium. The final concentration of each gut peptide in the media were 2 nM for the low dose, 20 nM for the intermediate dose and 100 nM for the high dose. The media used for the cells as a control had their pre-incubation medium renewed. Cells were collected after 4 h or 24 h in 700 μl of TRI Reagent (Sigma). After the total RNA was isolated, it was treated with a DNA-free kit (Section 2.5.2). The concentration of RNA was measured by a spectrophotometer (Section

2.5.3), and 1 μg of RNA was reverse transcribed to cDNA using the Reverse-iT First Strand Synthesis Kit (Section 2.6.).

The cDNA samples from treated groups and the control group were analysed by real-time PCR using the qPCR Core kit and the Mx3005P^{TM} QPCR System. The mRNA levels of each quantified gene were normalized to the value of human β actin, and the results were expressed as relative fold changes to control using the $^{2-\Delta\Delta}Ct$ method (section 2.10.3.4).

4.2.2 Statistics

Data are presented as mean values ± S.E.M. Differences between groups (treatments vs. control) were analysed by one-way ANOVA with Dunnett's post-hoc test. All data were checked for normality. There were no differences in variance amongst groups. Differences were considered to be statistically significant when $P<0.05$.

4.3 Results

4.3.1 The effect of GIP on adipokine gene expression in human SGBS adipocytes

The SGBS adipocytes were used at day 14 post-induction. Three different concentrations of GIP were employed; 2 nM for the low dose, 20 nM for the intermediate dose and 100 nM for the high dose.

No effect was found on mRNA level in the case of leptin in both 4 h and 24 h of GIP treatment (Fig 4.1). However, with the 24 h treatment a 2-fold increase in adiponectin mRNA level was evident at the higher dose of GIP treatment, while there was no response to the low or the intermediate doses of GIP (Fig 4.2).

Moreover, GIP treatment for 24 h led to an increase in IL-6 gene expression, with a 2-fold induction of IL-6 mRNA level in response to low, intermediate and high doses of GIP, but there was no change with the 4 h treatment (Fig 4.3). Interestingly in a similar pattern, a 2-fold increase in MCP-1 mRNA level was detected at low, intermediate and high doses applied to the differentiated SGBS adipocytes after 24 h

GIP treatment (Fig 4.4).

4.3.2 The effect of GLP-1 on adipokine gene expression in human SGBS adipocytes

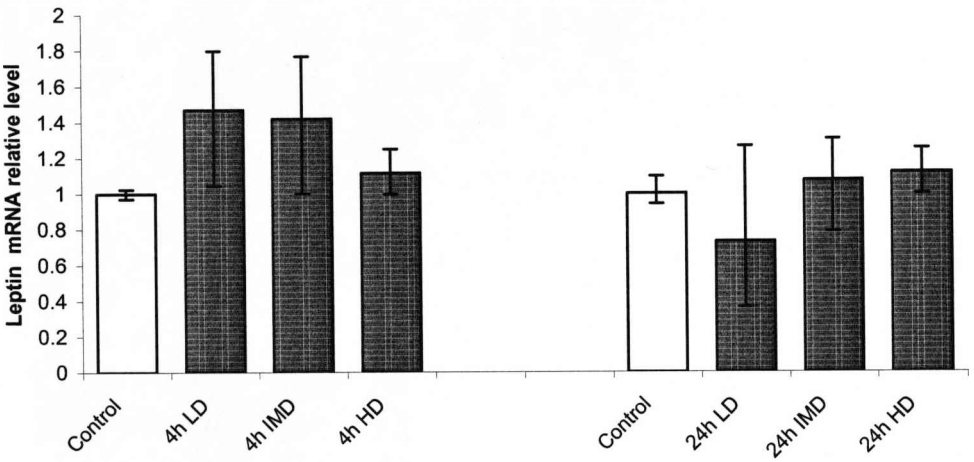
In the next set of experiments, the effect of GLP-1 on the production of important adipokines was examined in SGBS adipocytes. Administration of recombinant human GLP-1 for 4 or 24 h had no effect on leptin gene expression in SGBS cells (Fig 4.5). However, after applying GLP-1 for 24 h there was a dose-dependent increase in adiponectin gene expression, with a 2.5-fold and 4-fold induction of adiponectin mRNA level in response to the intermediate and higher dose respectively, but these was no change with the 4 h GLP-1 treatment in SGBS cells (Fig 4.6). A very small stimulatory effect of GLP-1 on IL-6 mRNA level was also observed at the higher dose only with both 4 and 24 h treatments, which caused a 70% increase (Fig 4.7). Whereas, neither the 4 nor 24 h GLP-1 treatment had any effect on MCP-1 gene expression in SGBS cells compared with the control (Fig 4.8).

4.3.3 The effect of ghrelin on adipokine gene expression in human SGBS adipocytes

To evaluate the effect of ghrelin on the key adipokines gene expression, SGBS adipocytes were treated with different concentrations of ghrelin for 4 and 24 h. Real time quantitative PCR analysis revealed that none of the doses of ghrelin influenced the mRNA expression of leptin, adiponectin and IL-6 gene expression (Fig 4.9, Fig 4.10; Fig 4.11).

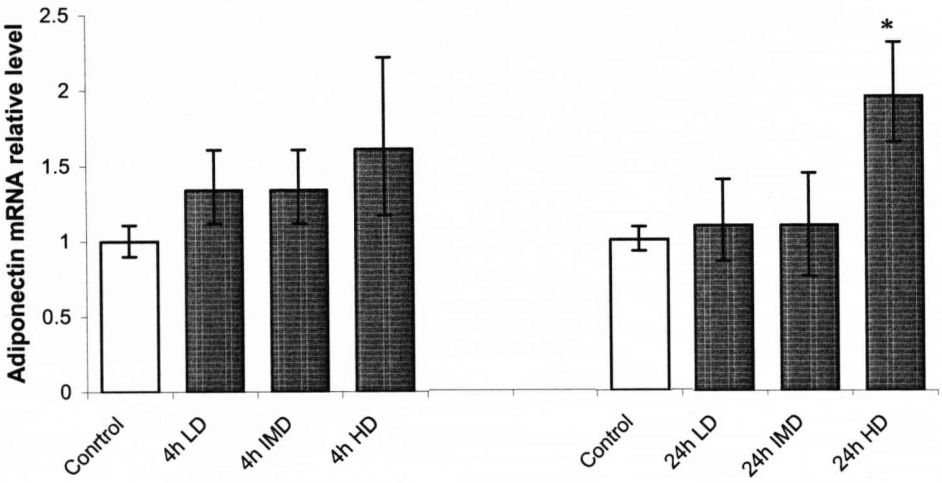
Interestingly however, ghrelin treatment for 24 h under the same conditions exerted an inhibitory effect on MCP-1 gene expression. MCP-1 mRNA levels were reduced by 50% with both intermediate and high doses (Fig 4.12); and these differences were statistically significant. However, MCP-1 mRNA level was not altered with the 4 h ghrelin-treated cells.

Figure 4.1 Effect of GIP on leptin gene expression in human SGBS adipocytes



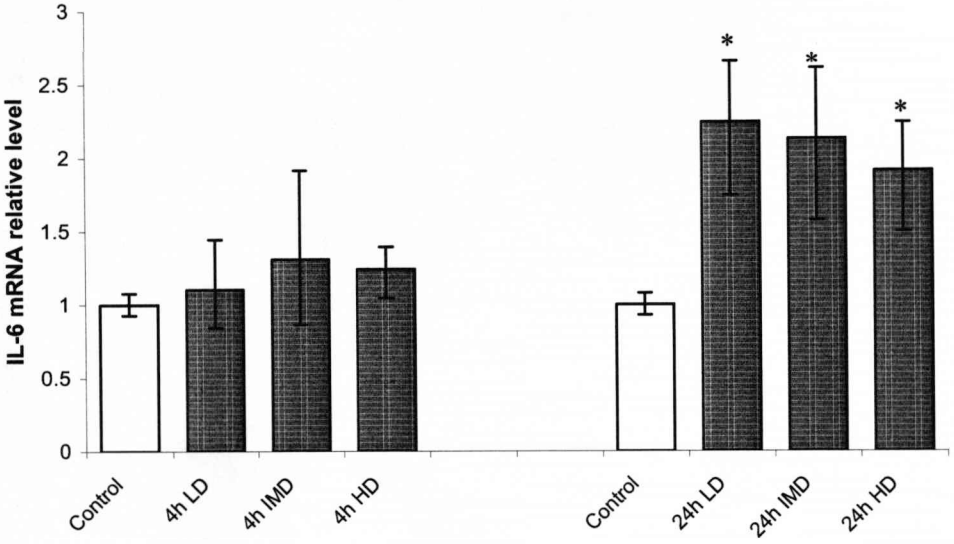
Effect of GIP on leptin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GIP for 4 and 24 h. Leptin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with GIP did not alter gene expession for leptin in both 4 h and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.2 Effect of GIP on Adiponectin gene expression in human SGBS adipocytes



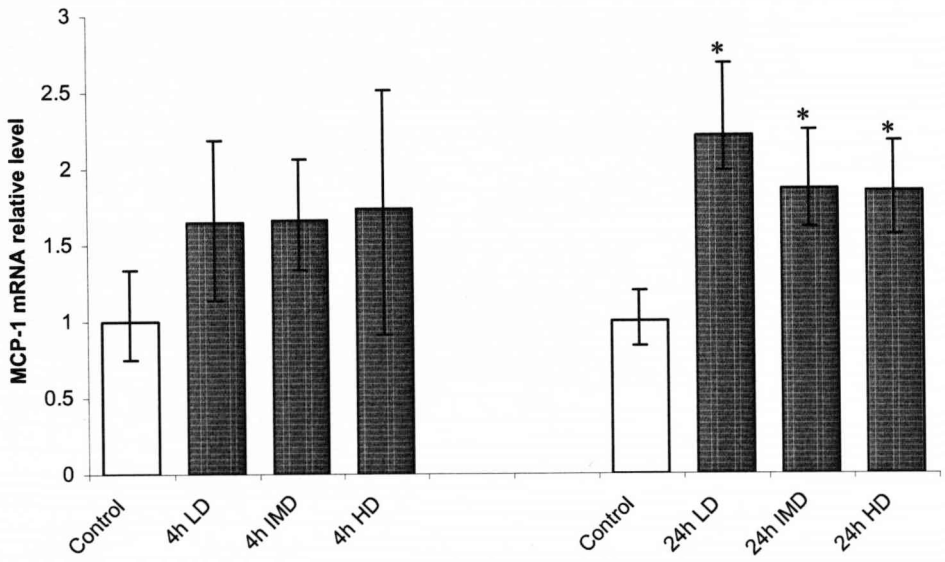
Effect of GIP on adiponectin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GIP for 4 and 24 h. Adiponectin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. The 24 h treatment at the higher dose of GIP showed a 2-fold increase in adiponectin mRNA. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 4.3 Effect of GIP on IL-6 gene expression in human SGBS adipocytes



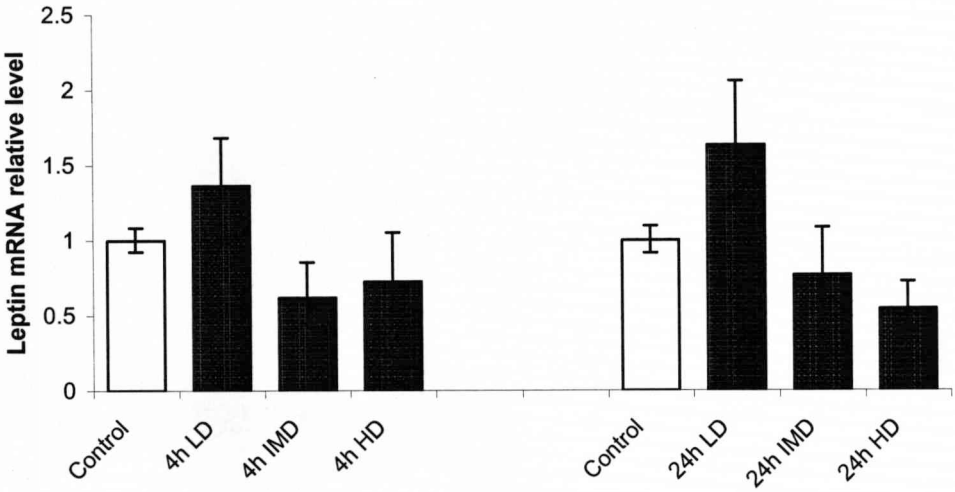
Effect of GIP on IL-6 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GIP for 4 and 24 h. IL-6 mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. GIP treatment for 24 h led to an increase in IL-6 gene expression however there was no change with the 4 h treatment. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 4.4 Effect of GIP on MCP-1 gene expression in human SGBS adipocytes



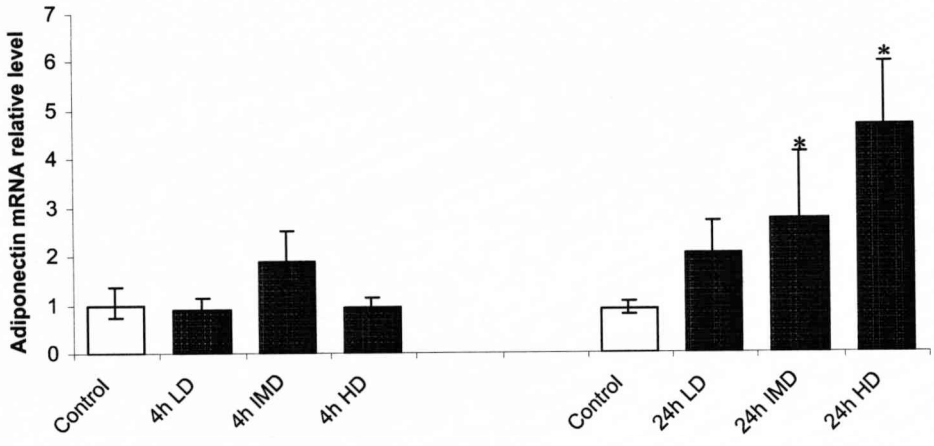
Effect of GIP on MCP-1 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GIP for 4 and 24 h. MCP-1mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. GIP treatment for 24 h led to an increase in MCP-1 gene expression but there was no change with the 4 h treatment. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 4.5 Effect of GLP-1 on leptin gene expression in human SGBS adipocytes



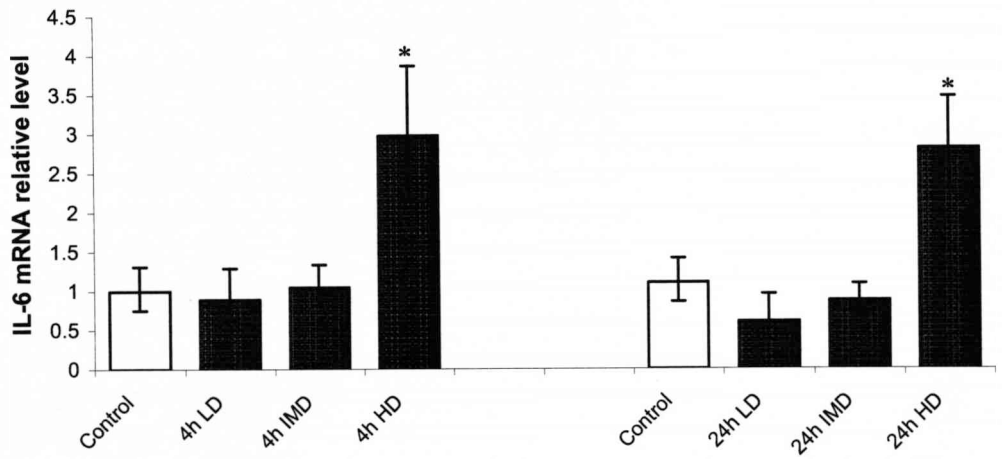
Effect of GLP-1 on leptin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GLP-1 for 4 and 24 h. Leptin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with GLP-1 did not alter gene expession for leptin in both 4 and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.6 Effect of GLP-1 on adiponectin gene expression in human SGBS adipocytes



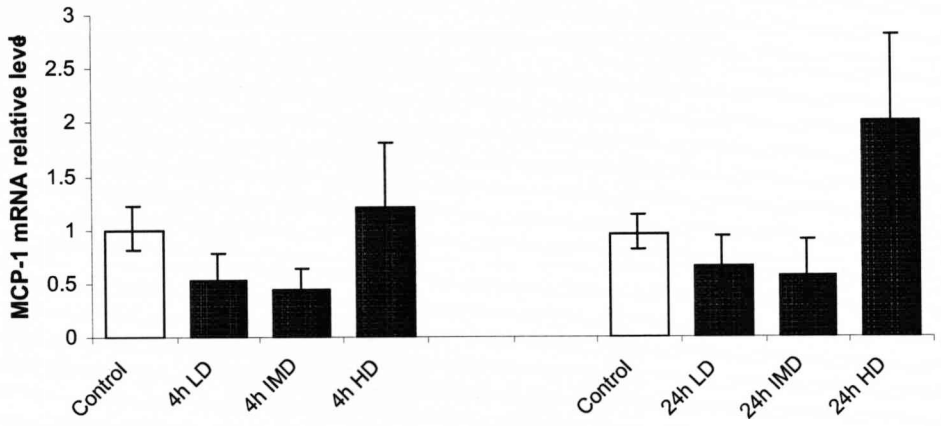
Effect of GLP-1 on adiponectin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GLP-1 for 4 and 24 h. Adiponectin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. There was a dose-dependent increase in adiponectin gene expression after applying GLP-1 for 24 h but there was no change with the 4 h GLP-1 treatment. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 4.7 Effect of GLP-1 on IL-6 gene expression in human SGBS adipocytes



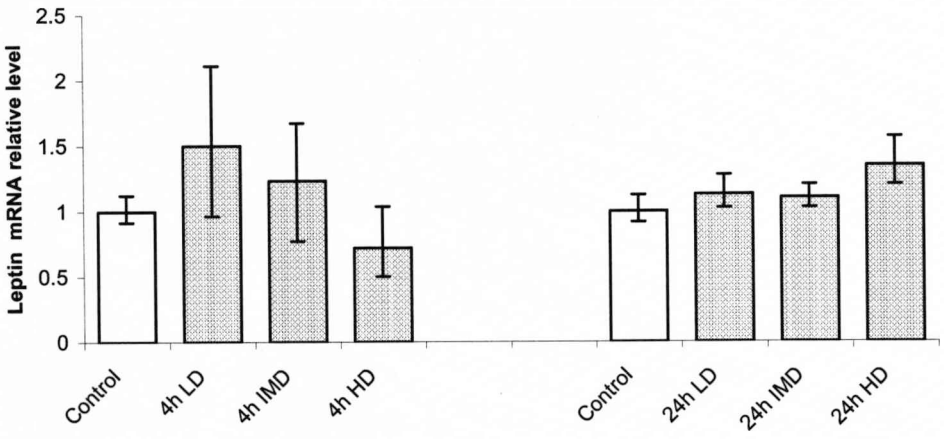
Effect of GLP-1 on IL-6 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GLP-1 4 and 24 h. IL-6 mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. A small stimulatory effect of GLP-1 on IL-6 mRNA level was observed only at the higher doses for both 4 and 24 h treatments. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 4.8 Effect of GLP-1 on MCP-1 gene expression in human SGBS adipocytes



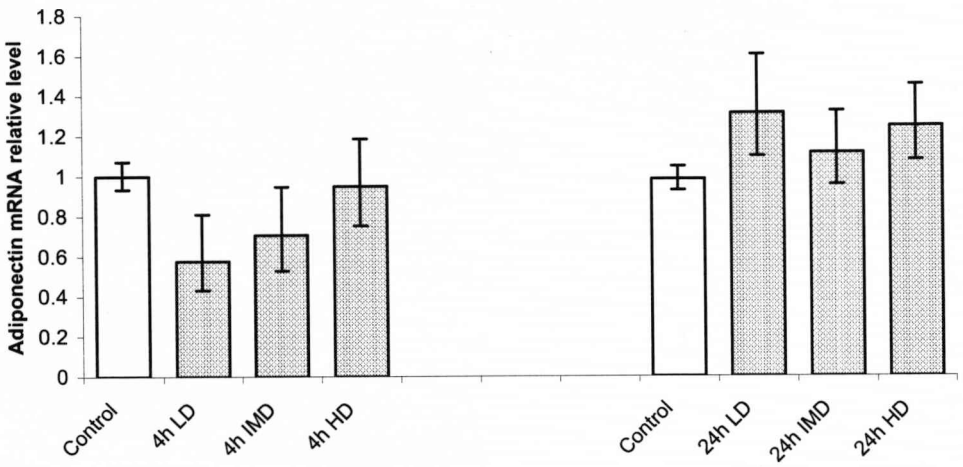
Effect of GLP-1 on MCP-1 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM GLP-1 4 and 24 h. MCP-1 mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with GLP-1 did not alter gene expession for MCP-1 at both 4 and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.9 Effect of ghrelin on leptin gene expression in human SGBS adipocytes



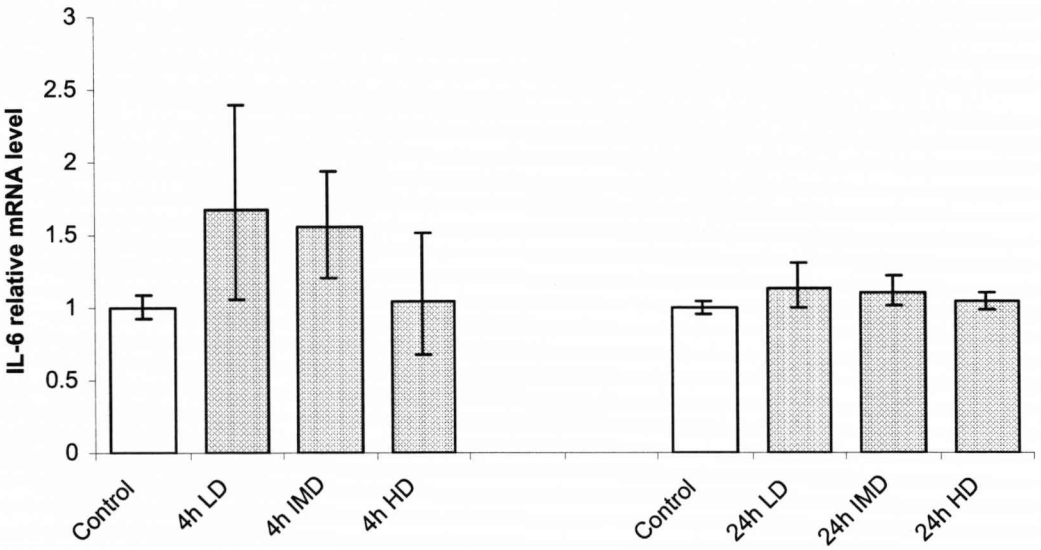
Effect of ghrelin on leptin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM ghrelin for 4 and 24 h. Leptin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with ghrelin did not alter gene expession for leptin at both 4 h and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.10 Effect of ghrelin on adiponectin gene expression in human SGBS adipocytes



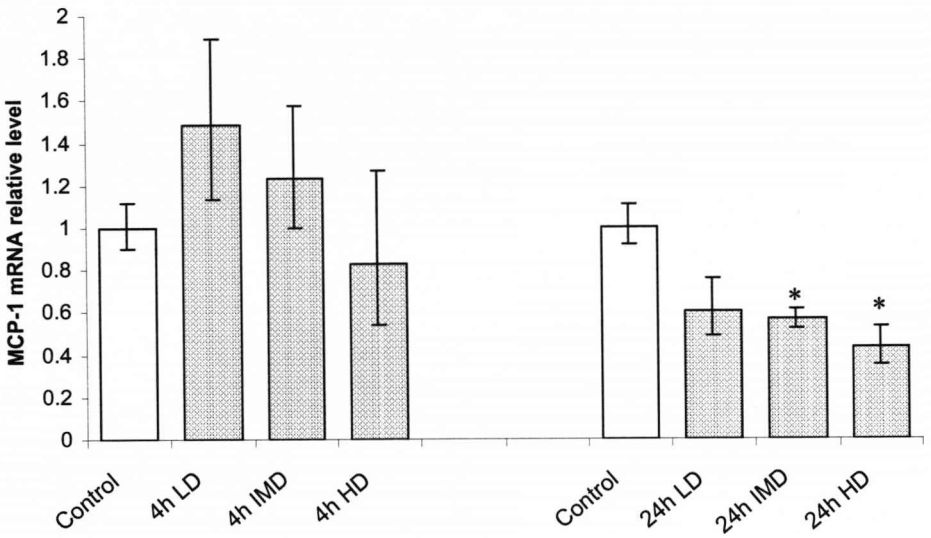
Effect of ghrelin on adiponectin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM ghrelin for 4 and 24 h. Adiponectin mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with ghrelin did not alter gene expession for adiponectin at both 4 h and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.11 Effect of ghrelin on IL-6 gene expression in human SGBS adipocytes



Effect of ghrelin on IL-6 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM ghrelin for 4 and 24 h. IL-6 mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with ghrelin did not alter gene expession for IL-6 at both 4 h and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 4.12 Effect of ghrelin on MCP-1 gene expression in human SGBS adipocytes



Effect of ghrelin on MCP-1 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM ghrelin for 4 and 24 h. MCP-1 mRNA levels were measured by real-time PCR and normalised to human β actin relative to the untreated control group. Ghrelin treatment for 24 h exerted an inhibitory effect on MCP-1 gene expression. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

4.4 Discussion

The key adipokines investigated in this set of studies including adipocytes hormones, adiponectin and leptin, IL-6, and MCP-1 are known for their role either locally, peripherally, or centrally in a variety of physiological processes including food intake, energy balance, insulin action, lipid metabolism, and homeostasis (Frühbeck *et al.,* 2001; Rajala & Scherer, 2003; Trayhurn & Beattie, 2001). Moreover, there is accumulating evidence to suggest that IL-6 and MCP-1 are linked to inflammation and the inflammatory response (Rajala & Scherer, 2003). Leptin itself has also been linked with inflammation (Sarraf *et al.,* 1997), whereas the other major adipocyte hormone, adiponectin, has an anti-inflammatory effect (Yokota *et al.,* 2000). The major aim of the studies in this section therefore was to examine whether GLP-1, GIP and ghrelin influence the expression of inflammatory-related adipokine genes in human adipocytes.

The results obtained in the current studies indicate that neither the incretins, GLP-1 and GIP, nor the ghrelin have effect on the regulation of leptin synthesis. However, both GLP-1 and GIP appears to have a small stimulatory effect on adiponectin mRNA levels at higher concentrations. Adiponectin is one of the most abundantly expressed adipose-specific proteins (Maeda *et al.,* 1997), and it has been implicated in the pathogenesis of obesity and insulin resistance in rodents and in humans (Kim *et al.,* 2002). Adiponectin levels are decreased in obese states and increased in individuals losing weight (Kim *et al.,* 2002). Furthermore, it has been demonstrated that administration of adiponectin improves insulin resistance and fatty acid oxidation in mice (Yamauchi *et al.,* 2002; Berg *et al.,* 2001), and that disruption of the adiponectin gene results in diet-induced insulin resistance (Maeda *et al.,* 2002).

In addition to the most remarkable ability of incretins to excite insulin secretion in the presence of glucose stimulatory concentrations (Kreymann *et al.,* 1987), the incretins role in regulating glucose and lipid metabolism has been reported by others (Hauner *et al.,* 1988; Wang *et al.*, 1997). The present data reveals that incretins may modulate the production of adipocyte-derived adipokines, by increasing adiponectin mRNA level after applying high doses of either GLP-1 or GIP for 24 h in differentiated SGBS cells. However, It should be noted, that the effect of incretins on adiponectin

expression is very small, and more work including the influence of both incretins on adiponectin release by fat cells is needed in the future.

MCP-1 is expressed primarily by immune cells, but it is also produced by human adipocytes (Gerhardt *et al.,* 2001; Wang *et al.,* 2005). MCP-1 has been recognized as a crucial signal governing the infiltration of macrophages into WAT in obesity (Xu *et al.,* 2003). The increased secretion of MCP-1 by WAT in obesity (Bruun *et al.,* 2005) may contribute to the inflammatory response and insulin resistance locally in adipose tissue (Dahlman *et al.,* 2005). In the present study, the stimulation of MCP-1 by GIP indicates that GIP may be involved in the synthesis of this adipokine in adipocytes. However, This stimulatory effect contradicts the proposed anti-obesity function of GIP (Gault *et al.,* 2003). Recently, studies have witnessed the development of a substantial number of designer enzyme-resistant 'super GIP' molecules with potent insulinotropic and anti-diabetic properties (Gault *et al.,* 2003). Nevertheless, observations in transgenic GIP receptor deficient mice indicate that GIP directly links overnutrition to obesity, therein playing a vital role in the development of obesity and related metabolic disorders (Miyawaki *et al.,* 2002).

Moreover the present results with 24 h GIP treatment in human adipocytes, showed that IL-6, another pro-inflammatory cytokine, gene expression was stimulated in a similar pattern. This indicates that GIP peptide is a regulatory agent that may have a role in the inflammatory response in white adipose tissue.

Unlike GIP, the data in regard to GLP-1 treatment, showed that the MCP-1 mRNA levels in human cell culture system did not change. However, a very small increase (although statistically significant) in IL-6 mRNA levels was observed in the high dose of GLP-1, at both 4 and 24 h of treatment, whereas IL-6 mRNA was not altered at the lower doses. The data has demonstrated that in general the regulation of incretins, GIP and GLP-1, in SGBS adipocytes is multi-factorial, and most likely the result of a complex interplay of different hormones and cytokines.

Little has been observed with regard to the regulation of adipokine production by ghrelin. However, a recent study has examined the possibility that ghrelin directly suppresses adiponectin mRNA expression in brown adipocytes, (Ott *et al.*, 2002).

The data here does not suggest such a role in SGBS (white) adipocytes.

Interestingly, however, ghrelin treatment for 24 h showed an inhibitory effect on MCP-1 mRNA levels by 50% in both intermediate and high doses, and these differences were statistically significant. Recent studies suggest that plasma concentrations of ghrelin are reduced in obese patients (Tschop *et al.,* 2001). Moreover, obesity has been associated with elevated levels of pro-inflammatory cytokines (Das, 2001). The mechanisms responsible for enhanced pro-inflammatory cytokine production in obesity remain to be elucidated.

The present results were strengthened by an independent study in which ghrelin was shown to have a potent anti-inflammatory effect in human endothelial cells, likely mediated by inhibition of NF-κB activation (Li *et al.,* 2004). This finding raises the possibility that reduction in endogenous ghrelin could potentially contribute to the pro-inflammatory state and the increased incidence of atherosclerosis in obese patients. Another study showed that peripherally administered ghrelin blocked IL-1β-induced anorexia (Asakawa *et al.,* 2001) and produced a positive energy balance by promoting food intake and decreasing energy expenditure.

In summary, the present study has demonstrated that GIP may serve as a regulatory in the inflammatory response in human adipocytes. The results obtained in this study also indicates that ghrelin has an anti-inflammatory effect on pro-inflammatory cytokine expression (MCP-1) in SGBS cells. In addition treatment with different gut hormones like incretins, GIP and GLP-1, or ghrelin has a selective effect on the production of a number of adipokines, such as adiponectin, MCP-1 and IL-6, in human adipocytes. However, it should be noted, that the effect of few tests revealed subtle differences (P-values $P<0.05$ or $P<0.01$) when multiple comparisons were made. There is a possibility that some of these differences have arisen by chance (Type 1 error). More work including the influence of incretins and ghrelin on adipokine expression by fat cells is needed in the future using larger sample sizes to justify that the effect did not just happen coincidentally. Nevertheless, this might constitute an important element in the pathogenesis of obesity.

CHAPTER 5
EFFECT OF OBESTATIN ON ADIPOKINE GENE EXPRESSION IN HUMAN ADIPOCYTES

5.1 Introduction

In a previous Chapter it was established that the gene encoding the proposed receptor for obestatin (GPR39) is expressed in adipose and nonadipose tissues, including four major WAT depots (subcutaneous, gonadal, omental and mesenteric) of the mouse. It was also shown that the receptor is expressed in WAT (subcutaneous and omental) of human subjects and in adipocyte cell culture systems of mouse (3T3-L1) and humans (SGBS). The receptor mRNA and protein was determined by using RT- PCR and Western blotting methods respectively.

The studies reported in this Chapter focus on investigating the effects of obestatin (1–23) and the truncated form obestatin (11–23), on the gene expression of key adipokines in human adipocytes. In order to perform this study, adipocyte cell cultures were utilised.

Obestatin is a newly identified 23 amino acid gut peptide, which is associated with the ghrelin peptide (Zhang *et al.,* 2005). Although both peptides originate from the same precursor (preproghrelin), they have opposing physiological roles. Obestatin exerts anorexigenic effects by decreasing food intake, gastric emptying, jejunal motility and weight gain by binding to the G-protein coupled receptor, GPR39. Zhang *et al.,* (2005) proposed GPR39 as the target receptor of obestatin, but this has not been confirmed by other studies (Lauwers *et al.,* 2006). In contrast, ghrelin (Kojima *et al.,* 1999) has positive effects on energy balance, stimulating food intake, gastric acid secretion, gastrointestinal motility and increasing feelings of hunger in humans. Both peptide hormones require post-translational modification for full activity; for obestatin, post-translational amidation occurs at the C-terminus.

In the original obestatin study by Zhang *et al*, mass spectrometry analysis suggested that in addition to the obestatin peptide peak of 2516.3 kD, another peak was eluted. This had a molecular mass of 1.5 kD and represented the last 13 residues of amidated obestatin. Now known as truncated des (1-10) obestatin analogs, or obestatin (11-23), it exhibits lower affinity binding to GPR39 when compared to obestatin (Zhang *et al.,* 2005).

The direct effects of obestatin on peripheral tissues that may contribute to the regulation of body weight and energy homeostasis are controversial. Since white fat mass is a target organ in obesity, it was tempting for researchers to speculate on whether obestatin is one of the factors responsible for the regulation of storage and mobilisation of body fat mass that might interfere with the overweight condition. Several factors have been implicated in the regulation of adipocyte number. Insulin, IGF-1, PPAR-γ ligands and corticosteroids have been found to increase adipocyte number, whereas TNF-α and leptin have been shown to decrease the number of these cells (Prins and O'Rahilly, 1997).

Tnag *et al.* have recently evaluated the effect of obestatin on cell proliferation in primary cultures of piglet adipose cells. The results showed that this peptide induced cell proliferation in a dose-dependent manner with MEK/ERK 1/2 phosphorylation (Tnag *et al.,* 2008). However, in another study obestatin did not rouse the cell cycle or viability of the murine adipocytes cell line 3T3-L1, other than by inhibiting the proliferation and differentiation of 3T3-L1 preadipocytes. Furthermore, the effect of obestatin was contrary to that of ghrelin on this adipocyte cell line (Zhang *et al.,* 2007). These findings suggest that direct effects of obestatin on proliferation and or differentiation in adipocytes may play a role in regulating body fat mass.

In general, little is known about the regulation of adipokine production by gut peptides. Moreover, the effect of obestatin on the regulation of adipokines has not been explored to date. In this set of studies, recombinant obestatin (1–23) and the truncated form, obestatin (11–23), was used to investigate the possibility that these peptides influence the expression of key adipokine genes in adipocytes. The effect of obestatin on the gene expression of leptin, adiponectin, MCP-I and IL-6 was therefore investigated in SGBS cells, as presented in the following section.

5.2 Methods

5.2.1 SGBS cell culture

SGBS cells were cultured as described in section 2.4.2. The preadipocytes were maintained in culture medium and differentiation of the cells was initiated 24 h after post-confluence by incubation for 4 days in induction medium. The cells were

maintained in feeding medium, which was renewed 2-3 times a week. At day 11, the cells were pre-incubated with cortisol and T_3- free feeding medium for 24 h. The human recombinant obestatin peptides explored in this set of studies were dissolved in 100% DMSO according to the protocol provided for this peptide, and the concentration of stock solutions for the peptide was 1 μM. Prior to treatment, incubation media were prepared by adding the peptide stock to the cortisol and T_3-free feeding medium. The final concentration of obestatin peptide in the media was 2 nM for the low dose, 20 nM for the intermediate dose and 100 nM for the high dose. The media used for the cells as a control had their pre-incubation medium renewed. Cells were collected after 4 or 24 h in 700 μl of TRI Reagent (Sigma).

In a separate set of studies, SGBS cells were incubated with human recombinant obestatin (1-23) from different sources (Peptides International, Peptide Institute or Alexis Biochemicals) as well as the truncated form, obestatin (11–23) (Peptide International). All peptides used in this set of studies were dissolved either in 100% DMSO, acetic acid or distilled water according to the protocol provided for each peptide. The final concentrations of each peptide in the media were 2 nM for the low dose and 100 nM for the high dose. The media used for the cells as a control had their pre-incubation medium renewed. Cells were then collected after 24 h in 700 μl of TRI Reagent.

5.2.2 Electrospray ionization mass spectrometry (ESI-MS)

The mass spectrometry study was kindly carried out by Mr Brian Green (VG BioTech/Fisons, Altrincham, England) using a VG BioQ mass spectrometer with electrospray (ES) ion source (section 2.13).

Initially, the obestatin amide sample was diluted 10-fold with water to give a stock solution. An aliquot of the stock solution was made up into aqueous acetonitrile solutions (50:50 v/v)/ 2% formic acid, with a final concentration of 25-50 pmol/ μL. Generally, a 10 μl aliquot of the analyte solution was injected, via a loop injector, into a stream of the same solvent mixture flowing at a rate of 5 μl /min. The mass spectrometer was then used to routinely scan over an appropriate m/z range determined experimentally. Generally several scans ≤15 were summed to obtain the

final ESI-MS spectrum. Calibration of the *m/z* scale of the spectrometer was normally performed during each series of measurements using a solution of pure protein of known structure.

5.2.2 Statistics

Data are presented as mean values ± S.E.M. Differences between groups (treatments vs. control) were analysed by one-way ANOVA with Dunnett's post-hoc test. All data were checked for normality. There were no differences in variance amongst groups. Differences were considered to be statistically significant when $P<0.05$.

5.3 Results

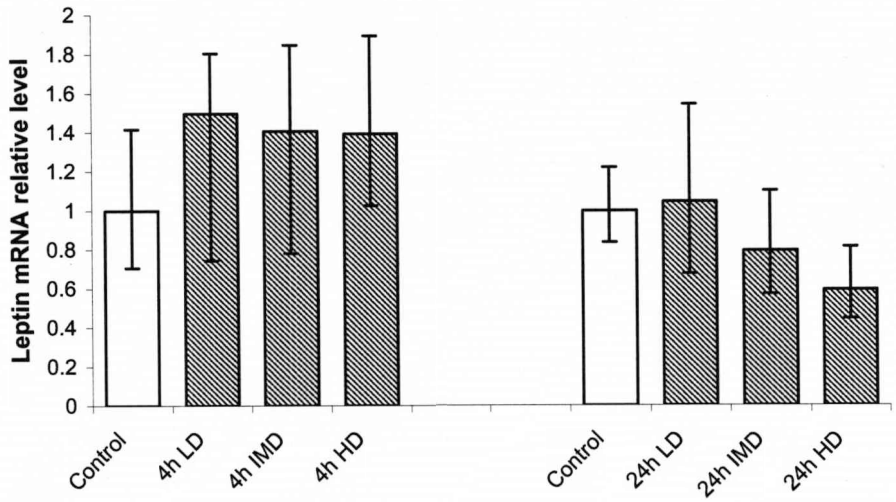
5.3.1 The effect of Obestatin (Alexis) on adipokine gene expression in human SGBS adipocytes

In the first studies, the integrated effect of obestatin on the expression of the adipokine genes was examined in differentiated SGBS adipocytes, with relative mRNA levels quantified by real-time PCR. The adipocytes were used at day 14 post-induction. Three different concentrations of obestatin were employed, 2 nM for the low dose, 20 nM for the intermediate dose and 100 nM for the high dose.

No effect was found on mRNA levels in the case of leptin at both 4 and 24 h of obestatin treatment (Fig 5.1). However, the 24 h obestatin treatment led to a dose-dependent decrease in adiponectin gene expression, with a 2-fold reduction of adiponectin mRNA levels in response to the intermediate and higher dose of obestatin, but there was no change seen after the 4 h obestatin treatment in SGBS cells (Fig 5.2).

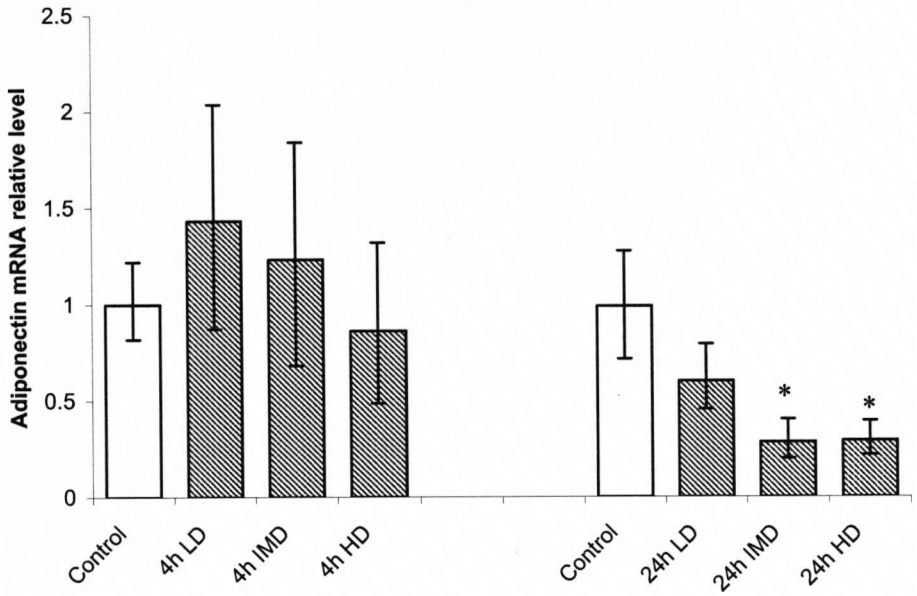
In contrast, treatment with obestatin resulted in a marked increase in mRNA levels for both IL-6 and MCP-1 (Fig 5.3 and 5.4). The increase was approximately 10-fold, 15-fold and 20-fold for IL-6 in 4 h low, intermediate and high doses respectively, and 10-fold in the 24 h obestatin treatments (Fig 5.3). The most substantial effect was on MCP-1 mRNA, the level of which was between 30 and 60-fold higher in both time courses (Fig 5.4).

Figure 5.1 Effect of obestatin on leptin gene expression in human SGBS adipocytes



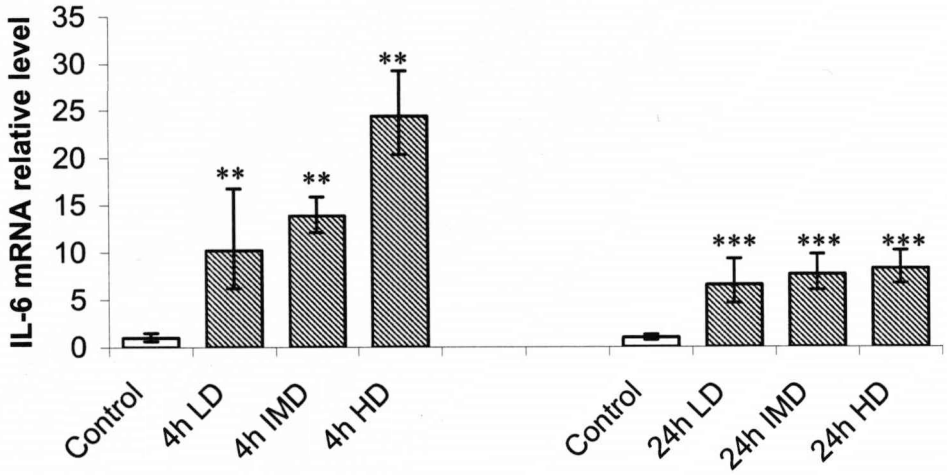
Effect of obestatin (Alexis) on leptin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestatin either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM for 4 h and 24 h. Leptin mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with obestatin did not alter gene expession for leptin at both 4 h and 24 h. Results are mean values ± S.E.M for groups of 5-6; compared with controls.

Figure 5.2 Effect of obestatin on adiponectin gene expression in human SGBS adipocytes



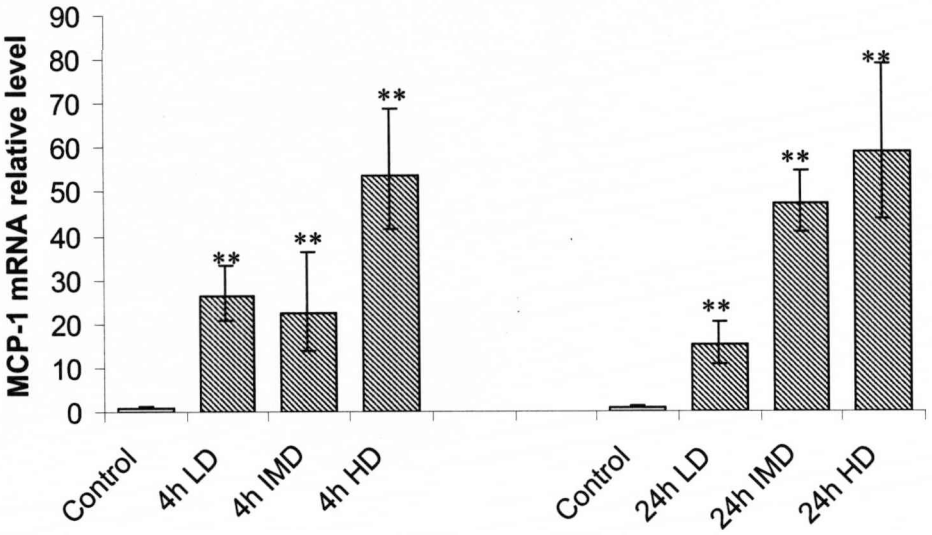
Effect of obestatin (Alexis) on adiponectin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestatin either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM for 4 h and 24 h. Adiponectin mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Obestatin treatment for 24 h exerted an inhibitory effect in adiponectin gene expression. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 5.3 Effect of obestatin on IL-6 gene expression in human SGBS adipocytes



Effect of obestatin (Alexis) on IL-6 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestatin either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM for 4 h and 24 h. IL-6 mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with obestatin resulted in a marked increase on IL-6 gene expression at both 4 and 24 h. Results are mean values ± S.E.M for groups of 5-6, **P<0.01, ***P<0.001; compared with controls.

Figure 5.4 Effect of obestatin on MCP-1 gene expression in human SGBS adipocytes



Effect of obestatin (Alexis) on MCP-1 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestatin either low dose (LD) 2 nM, intermediate dose (IMD) 20 nM or high dose (HD) 100 nM for 4 h and 24 h. MCP-1 mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Treatment with obestatin resulted in a marked increase on MCP-1 gene expression at both 4 and 24 h. Results are mean values ± S.E.M for groups of 5-6, **P<0.01; compared with controls.

5.3.2 The effect of obestatin (1-23) from different sources and des-obestatin (11-23) on adipokine gene expression in human SGBS adipocytes

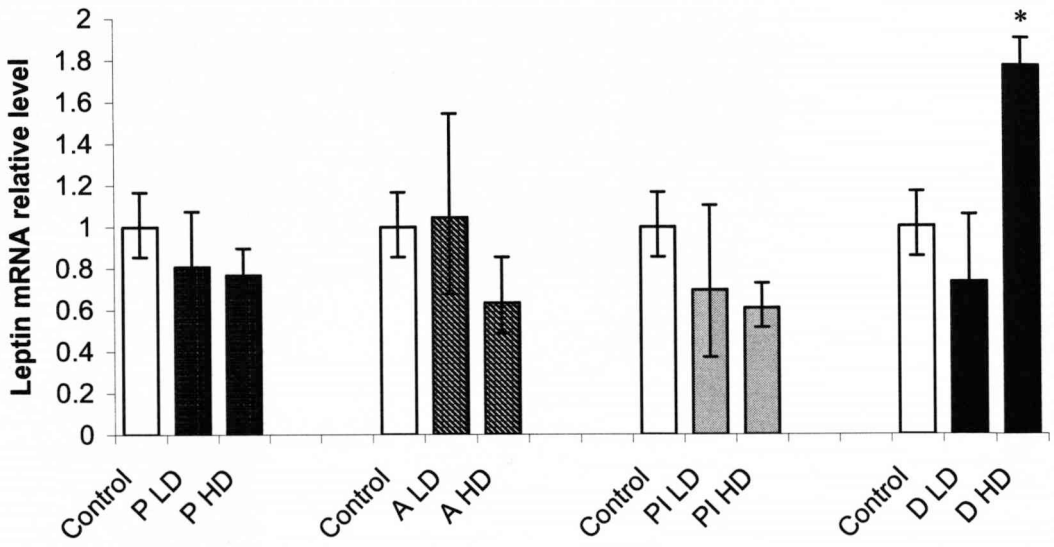
In a separate set of studies, SGBS cells were incubated for 24 h with human recombinant obestatin (1-23) from different sources: Peptides International (PI), Peptide institute (P), or Alexis Biochemicals (A), as well as the truncated form, des-obestatin (11–23) (D). The final concentrations of each peptide in the media were 2 nM for the low dose, and 100 nM for the high dose. The expression of the adipokine genes was examined in differentiated human adipocytes, with relative mRNA levels quantified by real-time PCR. Although two different concentrations of the obestatin(s) were employed, low and high, there was in practice little difference in the results obtained.

Administration of recombinant human (1-23) obestatin (s) had no effect on leptin gene expression in SGBS cells (Fig 5.5). However, the obestatin (1-23) treatments showed a 2-fold decrease in adiponectin mRNA levels and this was only present at the higher dose of all the (1-23) obestatin(s) used: there was no response to the low doses (Fig 5.6).

Moreover, after incubating SGBS with (1-23) obestatin the results were inconsistent with those shown in the previous section. Even when using the original obestatin (Alexis), there was only a modest stimulatory effect on both MCP-1 and IL-6 mRNA levels which was only observed at the higher doses, and caused only a 2 to 8-fold increase (Fig 5.7 and 5.8 respectively). In contrast, the low dose (1-23) obestatin (s) treatment had no effect on MCP-1 or IL-6 gene expression in SGBS cells compared with the control (Fig 5.7 and 5.8 respectively).

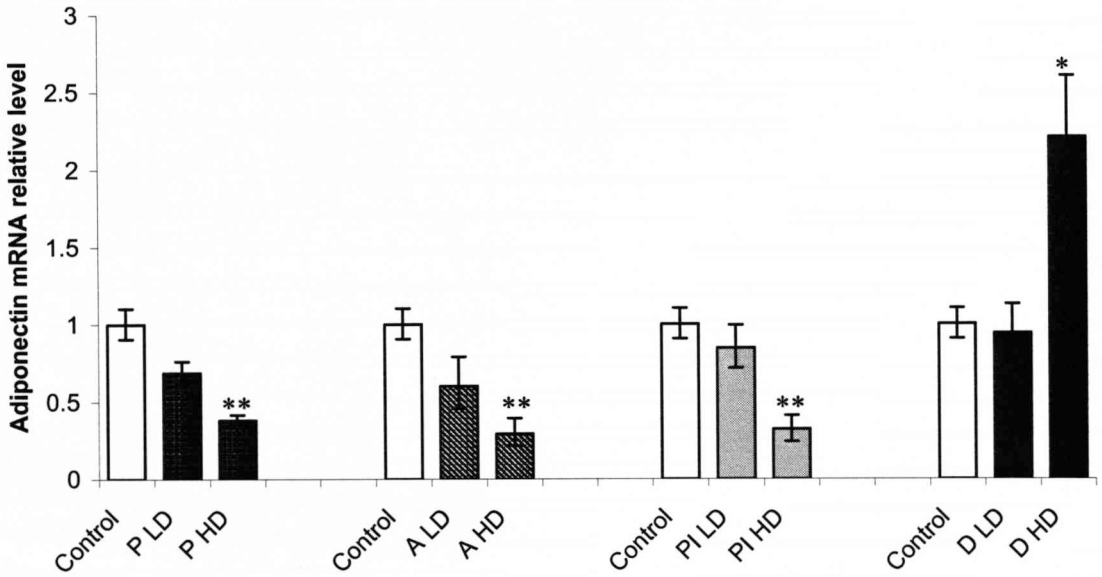
However, des-obestatin (11–23) showed a different response from those of the (1-23) obestatin. There was a marked increase in mRNA levels for leptin, adiponectin, IL-6 and MCP-1 (Figs 5.5, 5.6, 5.7 and 5.8). The increase was 2 to 3-fold in the case of leptin, adiponectin and MCP-1 and approximately 6-fold for IL-6 (Figs 5.5, 5.6, 5.7 and 5.8 respectively).

Figure 5.5 Effect of obestatin (1-23) from different sources and des-obestatin (11-23) on leptin gene expression in human SGBS adipocytes



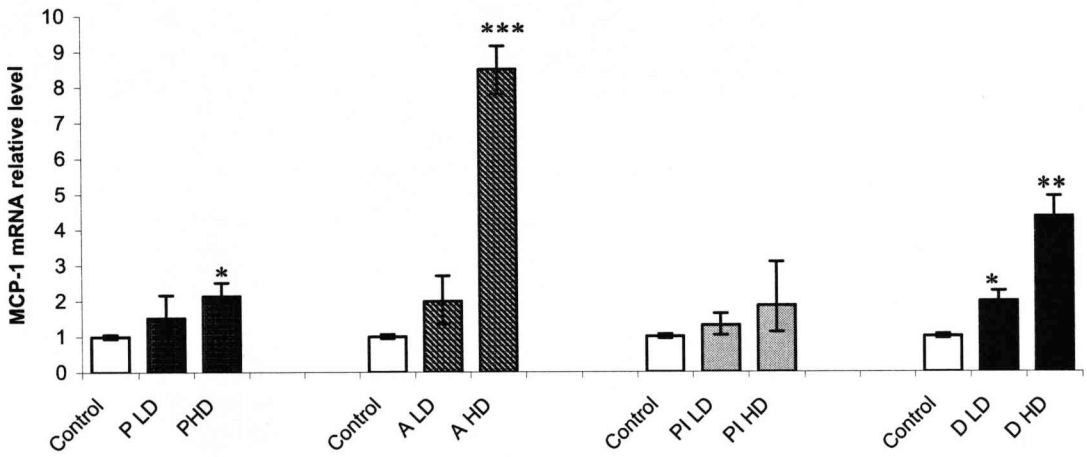
Effect of obestatin (1-23) from different sources, Peptide Institute (P), Alexis Biochemicals (A), Peptides international (PI) and des-obestatin (D) on leptin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestain either low dose (LD) 2 nM or high dose (HD) 100 nM for 24 h. Leptin mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Administration of recombinant human (1-23) obestatin (s) had no effect on leptin gene expression, however; des-obestatin showed a marked increase in mRNA levels for leptin at the high doses only. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

Figure 5.6 Effect of obestatin (1-23) from different sources and des-obestatin (11-23) on adiponectin gene expression in human SGBS adipocytes



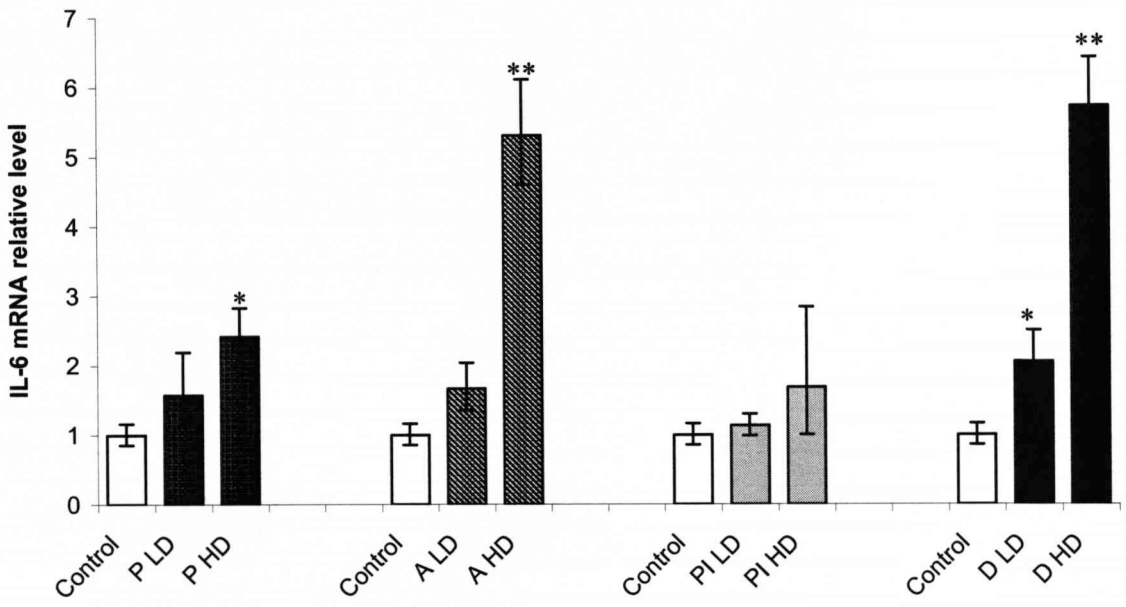
Effect of obestatin (1-23) from different sources, Peptide Institute (P), Alexis Biochemicals (A), Peptides international (PI) and des-obestatin (D) on adiponectin relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestain either low dose (LD) 2 nM or high dose (HD) 100 nM for 24 h. Adiponectin mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. The obestatin (1-23) treatments showed a clear decrease in adiponectin gene expression however; des-obestatin showed a marked increase in mRNA levels for adiponectin at the high dose only. Results are mean values ± S.E.M for groups of 5-6, *P < 0.05, **P<0.01; compared with controls.

Figure 5.7 Effect of obestatin (1-23) from different sources and des-obestatin (11-23) on MCP-1 gene expression in human SGBS adipocytes



Effect of obestatin (1-23) from different sources, Peptide Institute (P), Alexis Biochemicals (A), Peptides international (PI) and des-obestatin (D) on MCP-1 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestain either low dose (LD) 2 nM or high dose (HD) 100 nM for 24 h. MCP-1 mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Both obestatin (1-23) and des-obestatin groups showed a stimulatory effect on MCP-1 gene expression. Results are mean values ± S.E.M for groups of 5-6, *P < 0.05,**P<0.01, ***P<0.001; compared with controls.

Figure 5.8 Effect of obestatin (1-23) from different sources and des-obestatin (11-23) on IL-6 gene expression in human SGBS adipocytes



Effect of obestatin (1-23) from different sources, Peptide Institute (P), Alexis Biochemicals (A), Peptides international (PI) and des-obestatin (D) on IL-6 relative mRNA levels in SGBS cells. Differentiated human adipocytes at day 12 were deprived of cortisol and T_3 for 24 h and then incubated in medium containing obestain either low dose (LD) 2 nM or high dose (HD) 100 nM for 24 h. IL-6 mRNA level was measured by real-time PCR and normalised to human β actin relative to the untreated control group. Both obestatin (1-23) and des-obestatin led to an increase in IL-6 gene expression. Results are mean values ± S.E.M for groups of 5-6, *$P < 0.05$, **$P<0.01$; compared with controls.

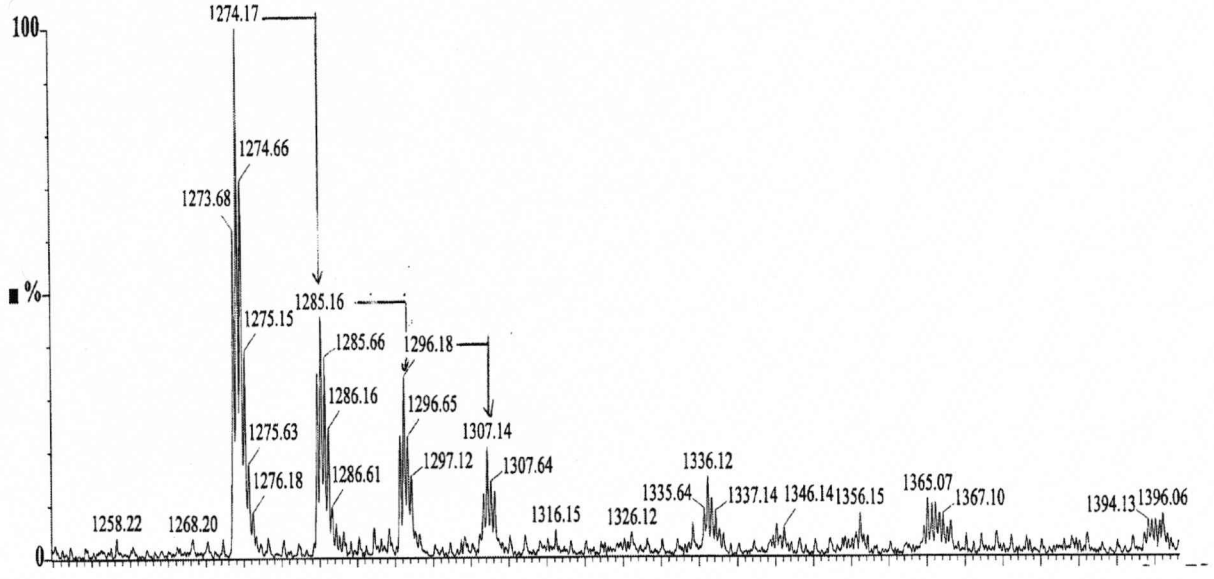
5.3.3 Obestatin detection by electrospray ionization mass spectrometry (ESI-MS)

Because of the inconsistency shown in the above set of results there was a question as why one specific obestatin (Alexis) behaving in an extreme way especially in regards with the production of the pro-inflammatory adipokines one possibility is that there were impurities which might lead to an inflammatory response. Therefore, to examine the purity of this specific sample the mass spectrometry study was performed and was kindly carried out by Mr Brian Green (VG BioTech/Fisons, Altrincham, England) using VG BioQ mass spectometer with electrospray (ES) ion source.

The obestatin amide sample was analysed by 10-fold diluting of the solution in 50% aqueous acetonitrile containing 0.2% formic acid. The result showed doubly and triply charged ions as major components that agreed with the elements composition within the experimental obestatin that calculated monoisotopic mass (Mr)= 2545.31. Moreover, the 2^+ ion gave m/z 1273.68, which implies Mr = 2545.34 and there were also 1, 2 and 3 sodium adducts associated with this ion (Fig 5.9). While the 3^+ ion gave m/z 849.48 which implies Mr = 2545.42 besides there was a DMSO adduct associated with this ion (Fig 5.10).

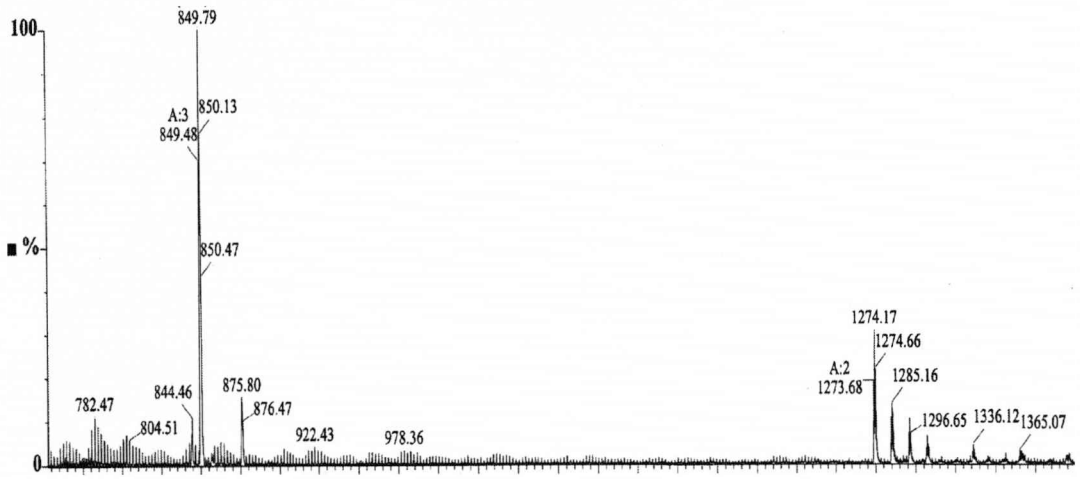
Furthermore, to ensure the absence of material-mediated pyrogenicity in the specific obestatin (Alexis), an endotoxin gel clotting technique was kindly performed by Mrs Janet Shaw (Dept of Microbiology, RLUH, Liverpool, England). The results showed that no endotoxin was detected at levels between 6 EU/ml and 60 EU/ml. This concludes that the results obtained for obestatin (Alexis) were not due to chemical extractables (bacterial endotoxins).

Figure 5.9 Obestatin detection by electrospray ionization mass spectrometry (ESI-MS)



Graph showing doubly charged ions and the major components that agreed with the elements composition within the experimental obestatin (Alexis), calculated monoisotopic mass (Mr)= 2545.31, which was analysed by mass spectrometry. The 2^{+} ion gave m/z 1273.68, which implies Mr = 2545.34, and there were also 1, 2 and 3 sodium adducts associated with this ion.

Figure 5.10 Obestatin detection by electrospray ionization mass spectrometry (ESI-MS)



Graph showing triply charged ions when analyzing the obestatin (Alexis) by mass spectrometry. The major components agreed with the elements composition within the experimental obestatin and the 3^{+} ion gave m/z 849.48 which implies Mr = 2545.42 besides there was a DMSO adduct associated with this ion.

5.4 Discussion

The major aim of the studies in this section was to examine whether obestatin influences the expression of key adipokine genes in human adipocytes. The results obtained indicate that obestatin has no effect on the regulation of leptin synthesis. However, obestatin appears to have a marked inhibitory effect on adiponectin mRNA levels after 24 h at the higher concentrations of obestatin administered.

This is the first study to demonstrate that obestatin triggers a robust sustained inhibition of adiponectin expression in differentiated adipocytes. Adiponectin production is suppressed in insulin resistance and obesity (Hu *et al.,* 1996; Weyer *et al.,* 2001), and its expression is regulated by hormones and drugs influencing insulin sensitivity (Maeda *et al.,* 2001). This suggests that obestatin could impair the insulin sensitivity by reducing adiponectin levels. A very recent *in vivo* study showed that fasting plasma concentration of obestatin is reduced in insulin resistance, and is positively associated with whole-body insulin sensitivity in non-diabetic humans. Furthermore, plasma obestatin is reduced by insulin in insulin-sensitive, but not in insulin-resistant persons (Anderwald-Stadler *et al.,* 2007). However another study reported that obestatin peptide had no effect on insulin sensitivity as revealed by hypoglycaemic response when mice were co-administered with insulin (Green *et al.,* 2007).

Initially in this study, obestatin had a strong stimulatory effect on the expression of two inflammation-related adipokine genes (MCP-1 and IL-6) in human adipocytes. The increase was approximately 20-fold and 15-fold for IL-6 in both 4 h and 24 h respectively. The most substantial effect was on MCP-1 mRNA, the level of which was almost 50 to 60-fold higher in both time courses.

However, the initial promise of a new regulator on pro-inflammatory adipokines was limited by further convergent studies negating these findings. In a subsequent set of studies, SGBS cells were incubated for 24 h with human recombinant obestatin (1-23) from different sources: Peptides International (PI), Peptide institute (P), or Alexis Biochemicals (A). The results showed a small stimulatory effect on both IL-6 and MCP-1 mRNA levels which was only observed at the higher doses. In contrast, the

low dose (1-23) obestatin (s) treatment had no effect on IL-6 or MCP-1 gene expression in SGBS cells compared with the control. Moreover, the results were inconsistent when repeated with the same obestatin (Alexis) as used in the previous section.

There is no exact explanation for the above results, especially after the certainty of the obestatin (Alexis) purity and pyrogenicity using mass spectrometry and endotoxin assays respectively. However, this study is one among many others that showed lack of reproducibility of the newly discovered gut peptide (Gourcerol. *et al.,* 2006; Holst *et al.,* 2007; Bassil *et al.,* 2007).

Nevertheless, it is emphasised that the present results with 24 h obestatin treatments from different sources in human adipocytes showed a stimulatory effect in both pro-inflammatory cytokine, MCP-1 and IL-6, mRNA levels in a similar pattern. This indicates that obestatin and des-obestatin peptides may act as regulatory agents that have a role in the inflammatory response in white adipose tissue. However, more work, including investigations on the influence of obestatin and des-obestatin on the inflammation-related adipokine release by fat cells, is needed in the future.

In this study whether the truncated form, obestatin (11–23), influences the gene expression of leptin, adiponectin, MCP-I and IL-6, in SGBS cells was also investigated. The results again were different from those of the (1-23) obestatin; however des-obestatin (11–23) showed a marked increase in mRNA levels for leptin, adiponectin, IL-6 and MCP-1. The increase was 2 to 3-fold in the case of leptin, adiponectin and MCP-1 and approximately 6-fold for IL-6.

Leptin is one of the best characterised adipokines and its expression and secretion are regulated by numerous factors, notably the activation of SNS, glucocorticoids, PPARγ agonists and insulin (Fain *et al.,* 2000; Havel, 2000; Rayner & Trayhurn, 2001). The data presented in this study have suggested that production of leptin is induced in response to des-obestatin (11–23) but not to obestatin (1-23) administration, although this effect is small.

Leptin has a pivotal role in the modulation of food intake and energy expenditure. Previous studies have shown that exogenous leptin administration results in the reduction of body fat (Chen *et al.,* 1996) and the stimulation of lipolysis in cultured adipocytes of rodents (Wang *et al.,* 1999). The present study may indicate a certain relationship between leptin and obestatin (1-23) or obestatin (11-23). However, a very recent *in vivo* study reported that plasma obestatin concentrations are negatively correlated with body mass index, insulin resistance index, and plasma leptin concentrations in obese humans (Nakahara *et al.,* 2007). Of course, des obestatin is a small fragment of obestatin, and may be part of a system with multiple effector elements, which not only have opposite actions, but also regulate the action of each other.

In summary, the present study has demonstrated that obestatin (1-23) and obestatin (11-23) may have a regulatory effect on the production of a number of adipokines, such as adiponectin, IL-6, MCP-1 and in the case of obestatin (11-23), leptin. However, it should be noted, that the effect of few tests revealed subtle differences (P-values $P<0.05$ or $P<0.01$) when multiple comparisons were made. There is a possibility that some of these differences have arisen by chance (Type 1 error). More work including the influence of obestatin on adipokine expression by fat cells is needed in the future using larger sample sizes to justify that the effect did not just happen coincidentally.

CHAPTER 6

DO GUT HORMONES ENHANCE INSULIN-STIMULATED GLUCOSE METABOLISM IN HUMAN ADIPOCYTES?

6.1 Introduction

Disordered glucose metabolism is commonly observed in obesity. Kinetic studies of glucose utilization in obese patients have demonstrated a reduction in the disappearance rate and uptake of glucose from the blood.

There is now considerable evidence in support of the concept that the disordered glucose metabolism of obesity may be related to the presence of insulin resistance. Studies on the effects of obesity on adipose tissue glucose metabolism and perfusion *in vivo* have however given controversial results. When radioactive glucose was given with glucose either intravenously (Björntorp *et al.*,1971) or orally (Mårin *et al.*, 1987) its incorporation into adipose tissue was 1–4% of glucose given in lean individuals, and the incorporation was increased in the obese subjects (up to 20%)(Björntorp *et al.*,1971; Mårin *et al.*, 1987).

The amount of free glucose in the body is small, typically around 12 g in the circulation and extravascular space. When a meal containing 100 g carbohydrate is ingested, the influx of glucose could potentially increase the plasma glucose concentration eightfold. This does not happen, because coordinated mechanisms come into play to increase the disposal of glucose from plasma and to suppress the entry into the circulation of endogenous glucose (Féry *et al.*, 1990). By these means, the exposure of tissues to hyperglycaemia are minimised. The main tissue involved in 'buffering' the influx of glucose is the liver, absorbing the glucose and switching off glucose production. Skeletal muscle and to a lesser extent adipose tissue play a subsidiary role in taking up glucose under the influence of insulin.

After food ingestion, the digestion and absorption of nutrients is associated with increased secretion of multiple gut peptides that act on distant target sites to promote the efficient uptake and storage of energy. Plasma concentrations of most gut hormones rise briskly within minutes of nutrient intake and fall rapidly thereafter, mainly because they are cleared by the kidney and are enzymatically inactivated.

Incretin hormones, GIP and GLP-1, which cause an increase in the amount of insulin released from the β cells of the islets augment the magnitude of meal-stimulated

insulin secretion from islet β cells in a glucose-dependent manner (Drucker, 2006). Incretin action facilitates the uptake of glucose by muscle tissue and the liver while simultaneously suppressing glucagon secretion by the α cells of the islets, leading to reduced endogenous production of glucose from hepatic sources.

Ghrelin also seems to have both physiological and pharmacological actions on the endocrine pancreas. Blocking the function of endogenous ghrelin with the use of an antagonist for its receptor markedly lowered fasting glucose concentrations, attenuated glycaemic excursion, and enhanced insulin responses during a glucose tolerance test, suggesting an inhibitory role for ghrelin in the control of insulin secretion (Dezaki *et al.,* 2004).

The recent discovery of obestatin in (2005) means that many of its biological actions remain to be identified, including whether this peptide influences *in vivo* glucose homeostasis or insulin secretion. Since ghrelin raises blood glucose and lowers insulin secretion, a number of studies are investigating whether obestatin administration might exert opposite effects on glucose homeostasis and insulin secretion.

In Chapter 3 of this thesis, the presence of genes encoding the receptors for ghrelin (GHS-R), Obestatin (GPR39), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) were examined in major fat depots of both mouse and human and in differentiated adipocytes. The existence of these receptors in fat cells is highly suggestive of physiological effects mediated by these gut peptides in adipose tissue.

Adipocytes are exquisitely sensitive to insulin; among the classical responses to insulin are an increase in glucose uptake, lipogenesis, and cellular proliferation and differentiation (Klein *et al.*, 2002). Moreover, the transport and metabolism of glucose is central to the function of WAT and the tissue is known to express several members of the facilitative glucose transporter (GLUT) gene family (Wood *et al.,* 2003; Wood and Trayhurn 2003). One of these transporters, GLUT4, is responsible for the acute insulin-induced stimulation of glucose transport in 3T3-L1 adipocytes (Kaestner *et al.* 1991, Czech *et al.* 1992). In addition to its acute effects, prolonged exposure to insulin has detrimental effects on glucose transport. Exposure of 3T3-L1 adipocytes to insulin

or cAMP for 24 h causes a down-regulation of GLUT4, both at the mRNA and protein levels, and a decrease in insulin-mediated glucose transport (Kaestner *et al.* 1991, Flores-Riveros *et al.* 1993).

In this series of experiments, the effects of a 24 h treatment with GLP-1, GIP, ghrelin and obestatin in the absence or presence of insulin, on glucose uptake and on the levels of expression of the facilitative glucose transporter, GLUT4, have been examined in SGBS adipocytes. The aim was to ascertain if prolonged exposure to these gut peptides has any modulatory effects by itself or on insulin action in cultured fat cells.

6.2 Methods

6.2.1 SGBS cell culture

SGBS cells were cultured as described in section 2.4.2. The preadipocytes were maintained in culture medium and differentiation of the cells was initiated 24 h after post-confluence by incubating the cells for 4 days in induction medium. The cells were maintained in feeding medium, which was renewed 2-3 times a week. At day 11, the cells were pre-incubated with cortisol and T_3- free feeding medium for 24 h. All the human recombinant gut peptides explored in this set of studies were dissolved either in 100% DMSO or autoclaved water according to the protocol provided for each peptide, and the concentration of stock solutions for each peptide was 1 μM. Prior to treatment, incubation media were prepared by adding each peptide stock to the cortisol and T_3-free feeding medium. The final concentration of each gut peptide in the media was 2 nM for the low dose and 100 nM for the high dose. The media used for the cells as a control had their pre-incubation medium renewed. Cells were collected after 24 h in 700 μl of TRI Reagent (Sigma). After the total RNA was isolated, it was treated with a DNA-free kit (Section 2.5.2). The concentration of RNA was measured by a spectrophotometer (Section 2.5.3), and 1 μg of RNA was reverse transcribed to cDNA using the Reverse-iT First Strand Synthesis Kit (Section 2.6.). In this set of studies, GLUT-4 primers were kindly provided by Dr S Wood (University of Liverpool). Relative quantification of gene expression was measured by real-time PCR using SYBR Green (Core kit, Eurogentec) incorporating a melt curve analysis for each run. All samples were normalized to the values of human β-

actin, and the results were expressed as relative fold changes to controls using the $^{2-\Delta\Delta}Ct$ method (section 2.10.3.4). The QPCR products detected primer sequences are shown in table 2.3.

6.2.2 2-Deoxy-D-glucose uptake

A modification of a method by Bernier *et al.* (1988) was used for the measurements of 2-deoxy-D-glucose (2-DG) uptake as described in section 2.12. Briefly SGBS fibroblasts were grown and differentiated in 12-well culture plates as described in section 2.4. After 24 h incubation with pretreatment medium in the presence or absence of 100 nM of human recombinant GLP-1, GIP, ghrelin or obestatin treatments, cells were washed twice and incubated with 950 µl of KRH buffer containing 1% BSA for 3 h at 37 °C, 5% CO_2. This was followed by the addition of different concentrations of insulin (0.1, 1, 10 and 100 nM).

In a separate set of experiments GLP-1 (0.1, 1 and 10 nM), GIP (0.1, 1 and 100nM) or ghrelin (1, 10 and 100 nM) were added without prior use of insulin. The cells were then incubated for 1 h at 37 °C (no insulin added for the basal uptake). 2-DG was added to a concentration of 60 µM containing 0.2 µCi/well of 2-deoxy-D-[1-^{3}H] glucose (Sp. Act 315 GBq/mmol, GE Healthcare) for 5 min at 37°C, 5% CO_2. Uptake was stopped by the addition of 2 ml of ice-cold PBS containing 200 µM phloretin (Sigma). The cells were washed three times with PBS stop solution, and solubilised in 0.1 N NaOH for 10 min at 22 °C. The cell lysates were subjected to liquid scintillation counting using EcoScint A fluid (National Diagnostics). The uptake of 2-DG was measured in the absence and presence of 40µM cytochalasin B to correct for non-specific uptake.

6.2.3 Statistics

The statistical significance of the difference between the means of two groups with equal variance was assessed by Student's *t*-test. However, the alternate (Welsch) *t*-test was used for the groups with unequal variance. The statistical differences between more than two groups (treatments vs control) were evaluated through one-way ANOVA (Analysis of Variance) with Dunnett's post-hoc test. All data were checked

for normality, and that there was no difference in variance amongst groups. Differences were considered to be significant when $P<0.05$. All results are presented as mean ± standard error of the mean (SE).

6.3 Results

6.3.1 Effects of GLP-1 on glucose uptake in adipocytes

The interaction between GLP-1 and 2-deoxy-D-glucose uptake was examined in the experiments depicted in Fig 6.1A. SGBS adipocytes were incubated with (100 nM) GLP-1. Cells were then washed twice and incubated with KRH buffer. This was followed by the addition of different concentrations of insulin for 1 h at 37 °C (no insulin added for the basal uptake). There was significant dose-response increase in acute insulin-stimulated glucose uptake when compared with control cells (Fig. 6.1A). In the absence of insulin the glucose uptake was increased threefold with 100 nM GLP-1 treatment for 24 h when compared with control cells ($P<0{\cdot}001$). (Fig. 6.1A)

In a separate experiment, the SGBS cells were incubated with pretreatment medium for 24 h in the absence of GLP-1. Cells were then washed twice and incubated with the buffer, then different concentrations of GLP-1 (0.1, 1 and 10 nM) was added to the cells for 1 h without prior use of insulin. Compared with untreated cells, again there was significant dose-response increase 2-fold, 2-fold and 3-fold respectively, in glucose uptake (Fig 6.1B).

6.3.2 Effects of GIP on glucose uptake in adipocytes

In the subsequent studies, the effect of GIP on glucose uptake was examined in SGBS adipocytes. Administration of 100 nM GIP for 24 h in the presence of different concentrations of insulin had no effect on the glucose uptake compared with control cells (Fig. 6.2A).

The effect of GIP was further explored by monitoring its ability to enhance glucose uptake under basal conditions and in the absence of insulin. Different concentrations of recombinant GIP (0.1, 1 and 100 nM) were added to the SGBS cells for 1h without

the use of insulin. Compared with untreated cells, the glucose uptake was only induced by threefold with the 100 nM GIP treated adipocytes (Fig 6.2B); however, glucose uptake showed no changes in both 1 nM and 10 nM GIP treatment (Fig 6.2B).

6.3.3 Effects of ghrelin on glucose uptake in adipocytes

To examine the potential impact of ghrelin on glucose transport in differentiated SGBS adipocytes, 2-deoxy-D- glucose uptake assay was performed.
Applying 100 nM of ghrelin treatment for 24 h in the differentiated SGBS adipocytes significantly increased glucose uptake by fivefold in the absence of insulin. However after adding (0.1, 1, 10 and 100 nM) of insulin for 1 h to the treated cells; the glucose uptake was significantly down regulated compared with controlled untreated cells (Fig. 6.3A)

In a different experiment, pretreatment medium was applied to the SGBS adipocytes for 24 h in the absence of both ghrelin and insulin. Cells were then washed twice and incubated with the buffer, then different quantities of ghrelin (1, 10 and 100nM) were added to the cells for 1 h. Compared with untreated cells, the ghrelin augmented 2-deoxy-D-glucose uptake in SGBS adipocytes and was significant at both intermediate and high doses, the increases being three and four fold respectively ($P<0.001$). (Fig. 6.3B)

6.3.4 Effects of Obestatin on glucose uptake in adipocytes

The effects of a 24 h treatment with obestatin in the absence or presence of insulin on glucose uptake was measured in differentiated SGBS adipocytes.
Administration of recombinant 100 nM obestatin for 24 h in the presence of different concentrations of insulin (0.1, 1, 10 and 100 nM) showed a small significant increase (1.5-fold), in glucose uptake at 0.1 nM of insulin only (Fig. 6.4); however, glucose uptake showed no changes at the higher doses of insulin.

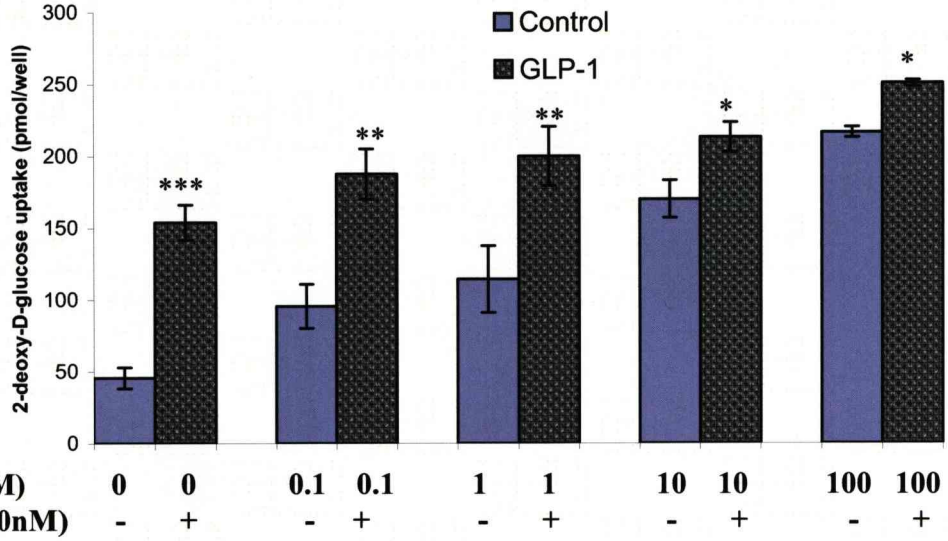
6.3.5 Effects of GLP-1or Ghrelin on GLUT4 mRNA expression

Based on the results above, both GLP-1 and ghrelin have a robust effect on glucose uptake. By using real-time PCR their impact on GLUT4 gene expression in SGBS adipocytes was next assessed as described in section 6.2.1.

In this set of experiments, GLUT4 gene expression was examined after 24 h incubation with the low (2 nM) and high (100 nM) doses of recombinant GLP-1 or ghrelin peptides. No treatment was added for the control group except their pre-incubation medium was renewed. A minor decrease of GLUT4 mRNA levels was only observed in the low dose of GLP-1 treatment (Fig 6.5), whereas GLUT4 mRNA was not altered at the high GLP-1 dose or with the ghrelin treatment.

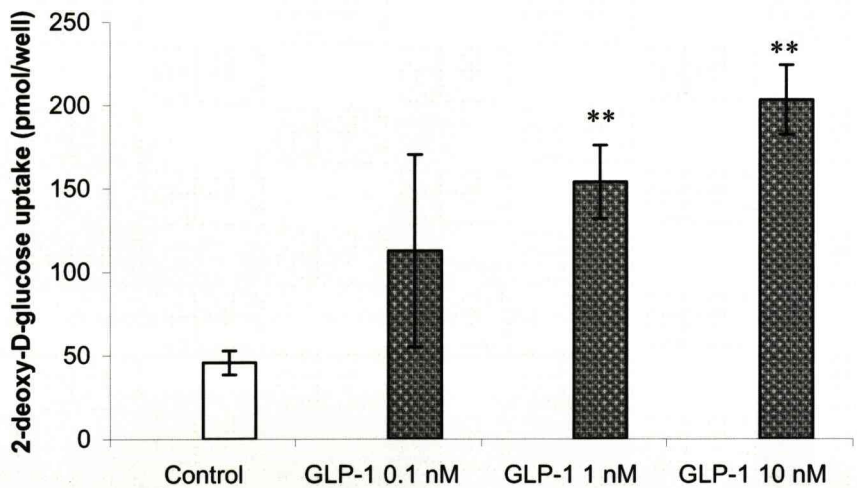
Figure 6.1 Effects of GLP-1 on glucose uptake in SGBS adipocytes

A



Effect of GLP-1 on 2-deoxy-D-glucose uptake in SGBS adipocytes at day 14 (postinduction of differentiation). Applying 100 nM of GLP-1 treatment for 24 h in the differentiated SGBS adipocytes significantly increased glucose uptake in the absence and presence of insulin (0.1, 1, 10 and 100 nM) for 1 h. The results were assessed by Student's *t*-test/ Welsch *t*-test and expressed as mean values ± SE, (n = 6). * $P < 0.05$; **$P < 0.01$, *** $P < 0.001$, compared to adipocytes without treatment with GLP-1.

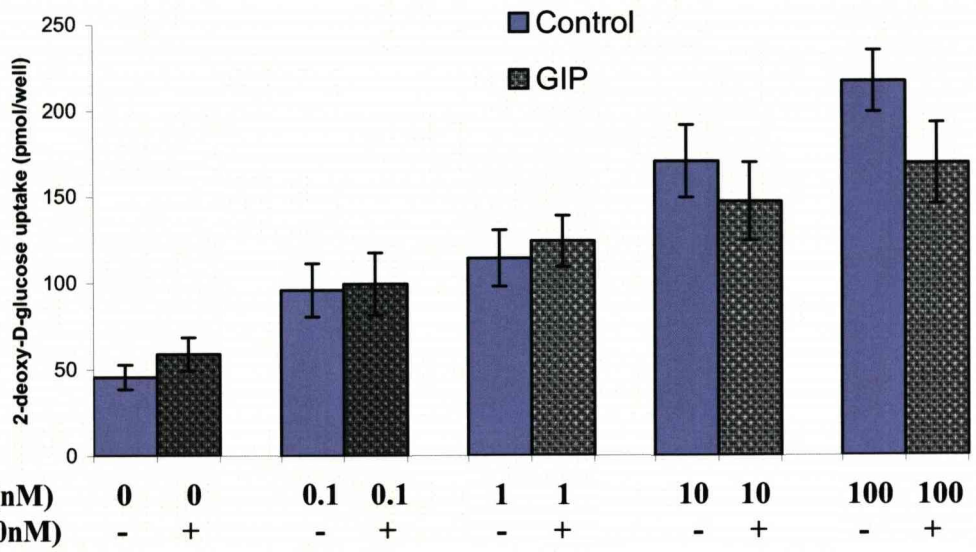
B



SGBS cells were incubated with pretreatment medium for 24 h in the absence of GLP-1. When different concentrations of GLP-1 (0.1, 1 and 10 nM) were added to the cells for 1 h without prior use of insulin a significant dose-response increase was shown on 2-deoxy-D-glucose uptake. The results were assessed by ANOVA and expressed as mean values ± SE, (n = 6). **$P < 0.01$; compared to adipocytes without treatment with GLP-1.

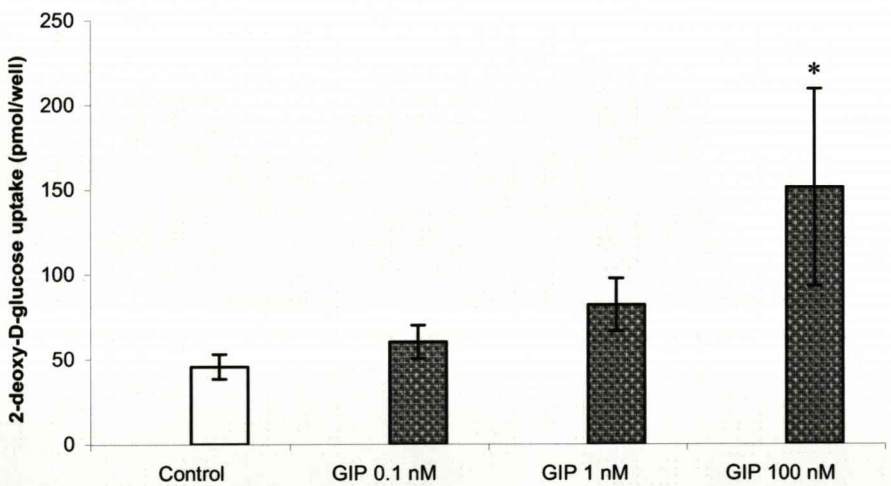
Figure 6.2 Effects of GIP on glucose uptake in SGBS adipocytes

A



Effect of GIP on 2-deoxy-D-glucose uptake in SGBS adipocytes at day 14 (postinduction of differentiation). Applying 100 nM of GIP treatment for 24 h in the differentiated SGBS adipocytes significantly increased glucose uptake in the absence and presence of insulin (0.1, 1, 10 and 100 nM) for 1 h. The results were assessed by Student's *t*-test/ Welsch *t*-test and expressed as mean values ± SE, (n = 6), compared to adipocytes without treatment with GIP.

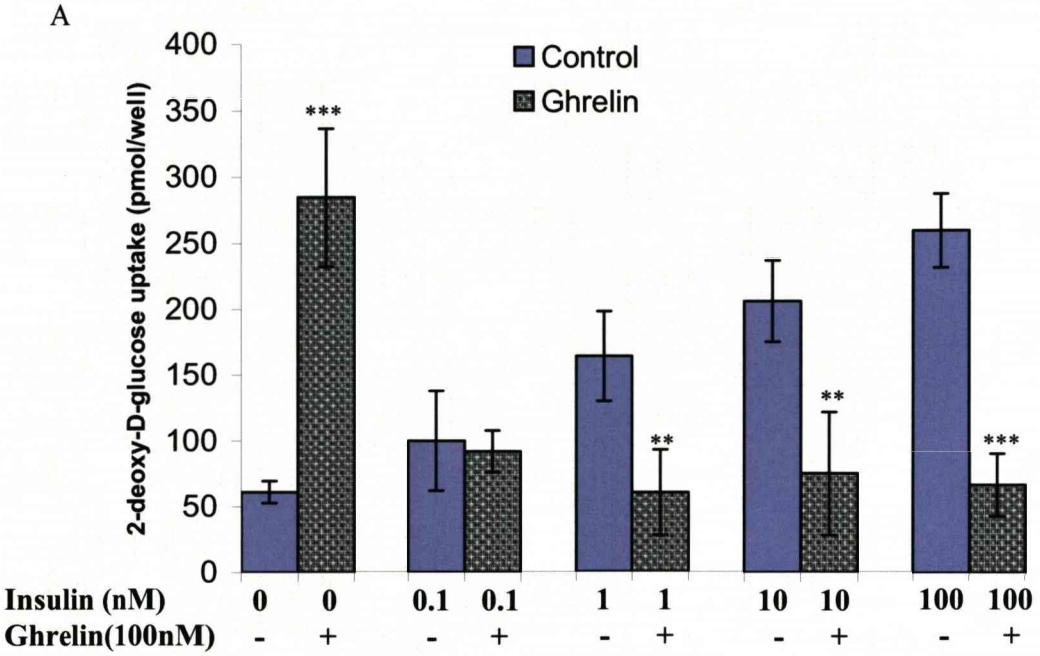
B



SGBS cells were incubated with pretreatment medium for 24 h in the absence of GIP. When different concentrations of GIP (0.1, 1 and 100 nM) were added to the cells for 1 h without prior use of insulin. The 2-deoxy-D-glucose uptake was only induced with the 100 nM GIP treated adipocytes. The results were assessed by ANOVA and expressed as mean values ± SE, (n = 6). *P < 0.05; compared to adipocytes without treatment with GIP.

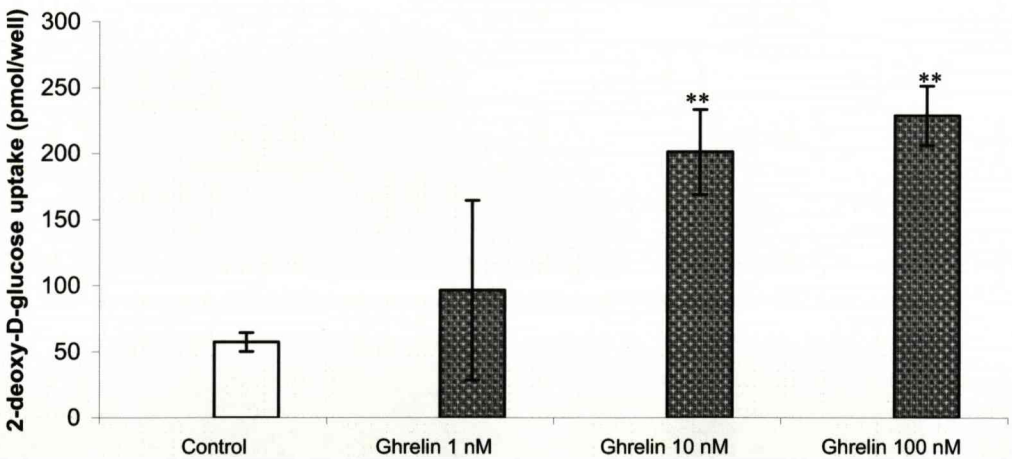
Figure 6.3 Effects of ghrelin on glucose uptake in SGBS adipocytes

A



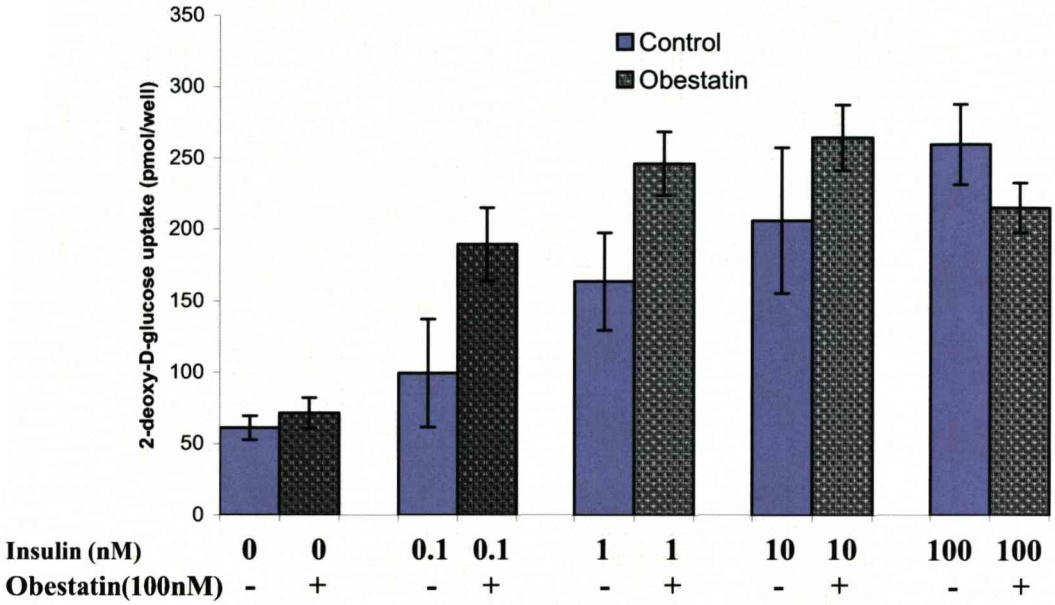
Effect of ghrelin on 2-deoxy-D-glucose uptake in SGBS adipocytes at day 14 (postinduction of differentiation). Applying 100 nM of ghrelin treatment for 24 h in the differentiated SGBS adipocytes significantly increased glucose uptake in the absence and presence of insulin (0.1, 1, 10 and 100 nM) for 1 h. The results were assessed by Student's *t*-test/ Welsch *t*-test and expressed as mean values ± SE, (n = 6). **P < 0.01, *** P < 0.001, compared to adipocytes without treatment with ghrelin.

B



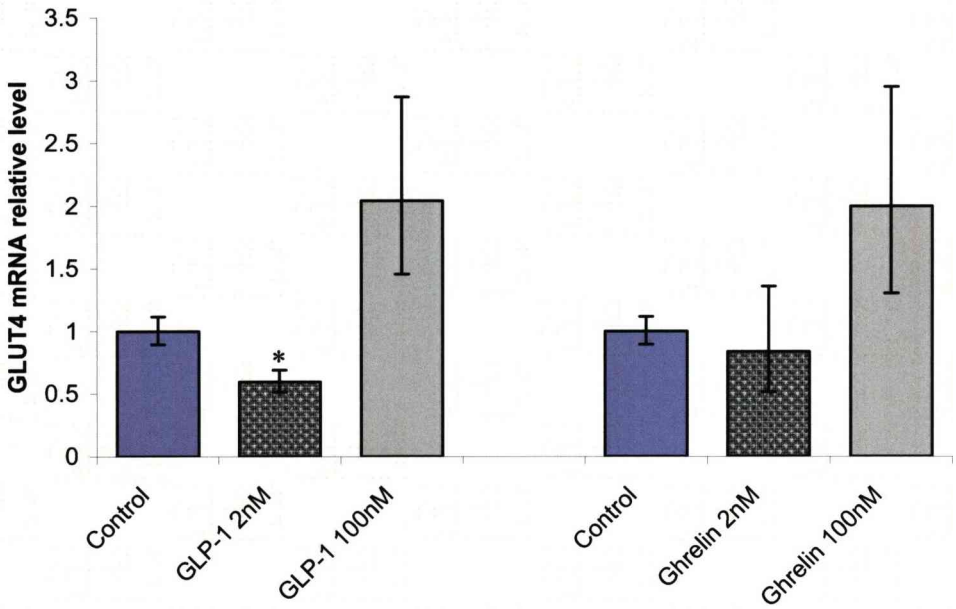
SGBS cells were incubated with pretreatment medium for 24 h in the absence of ghrelin. When different concentrations of ghrelin (1, 10 and 100 nM) were added to the cells for 1 h without prior use of insulin a significant dose-response increase was shown on 2-deoxy-D-glucose uptake. The results were assessed by ANOVA and expressed as mean values ± SE, (n = 6). **P < 0.01; compared to adipocytes without treatment with ghrelin.

Figure 6.4 Effects of obestatin on glucose uptake in SGBS adipocytes



Effect of obestatin on 2-deoxy-D-glucose uptake in SGBS adipocytes at day 14 (postinduction of differentiation). Applying 100 nM of obestatin treatment for 24 h in the differentiated SGBS adipocytes significantly increased glucose uptake in the absence and presence of insulin (0.1, 1, 10 and 100 nM) for 1 h. The results were assessed by Student's *t*-test/ Welsch *t*-test and expressed as mean values ± SE, (n = 6), compared to adipocytes without treatment with obestatin.

Figure 6.5 Effects of GLP-1or Ghrelin on GLUT4 mRNA expression



Effect of GLP-1 and ghrelin on glucose transporter 4 (GLUT4) gene expression in human SGBS adipocytes. A minor decrease of GLUT4 mRNA levels was only observed in the low dose of GLP-1 treatment, whereas GLUT4 mRNA was not altered at the high GLP-1 dose or with the ghrelin treatment. Cells were harvested at day 14 (post-induction of differentiation) and incubated for 24 h in media containing GLP-1 or ghrelin. Total RNA was isolated and GLUT4 mRNA levels quantified by real-time PCR. The values were normalised to β-actin and expressed relative to controls. The results were assessed by ANOVA and expressed as mean values ± S.E.M for groups of 5-6, *$P < 0.05$; compared with controls.

6.4 Discussion

Insulin, the most potent mammalian anabolic hormone (Saltiel and Kahn 2001), appears to have evolved from the need to maximise energy efficiency, obviating the requirement to forage continuously for food. Organisms expressing this important peptide hormone possessed a distinct survival advantage and flourished. During evolution, insulin biosynthesis translocated to the pancreas, possibly to protect β cells from enteric organisms, which then necessitated a messenger from the intestine to complete an entero-insular axis.

The eventual development of incretins (Tseng *et al.,* 1996; Wang *et al.,* 1995) and other recently discovered gut peptides fulfilled this requirement. The additional survival benefit offered by a specific gut hormone may have been the ability of those regulatory peptides to not only stimulate intestinal glucose absorption and maximally release insulin (Tseng *et al.,* 1999) but to also possess insulin mimetic properties.

In this chapter, the effects of a 24 h treatment with GLP-1, GIP, ghrelin and obestatin in the absence or presence of insulin, on glucose uptake and on the levels of expression of the facilitative glucose transporter, GLUT4, in fully differentiated SGBS adipocytes were investigated.

6.4.1 Effects of incretins on glucose uptake in SGBS adipocytes

At 24 h GLP-1 treated SGBS adipocytes, showed a clear dose-dependent increase in glucose uptake with or without acute insulin-stimulation when compared with control cells.

One would suspect that GLP-1 might enhance several metabolic responses of insulin in target tissues. This study with differentiated SGBS adipocytes has contributed significantly toward this end. A previous study revealed that GLP-1 has acute effects on glucose metabolism in 3T3-L1 adipocytes (Egan *et al.* 1994), in which the actions of insulin to stimulate glucose uptake and its utilization for lipid synthesis were enhanced by the presence of GLP-1. They also showed that GLP-1 preferentially increases lipid synthesis without increasing glycogen synthesis. Furthermore, *In vivo*

studies in humans have also shown that GLP-1 has effects on glucose disposal (D'Alessio *et al.*, 1994; Meier *et al.,* 2006).

The results of this set of experiments, therefore, demonstrated that in addition to stimulating insulin release from pancreatic β cells, GLP-1 possesses insulin-mimetic properties in adipocytes. Like insulin, GLP-1 induces the activation that leads to enhanced adipocyte glucose uptake.

Although the insulin-releasing action of GIP makes a major contribution to its metabolic role, direct effects of GIP at extra-pancreatic sites, including stomach, liver and adipose tissue, have been suggested to contribute to the overall action of the hormone on post-prandial nutrient homeostasis (Fehmann *et al.* 1995).

Earlier studies had shown that GIP promotes the incorporation of glucose into extractable lipids (Hauner *et al.,* 1988). In addition, GIP has been found to regulate the function of lipoprotein lipase, which hydrolyses chylomicrons and very-low-density lipoproteins, thereby liberating fatty acids for uptake and storage within the adipocyte (Murphy *et al.,* 1995). These studies indicate that GIP may represent a major hormonal signal linking meal content and size to postprandial lipoprotein lipase activity (Eckel *et al.,* 1979) and modulating circulating lipoprotein homeostasis.

The addition of 100 nM of GIP in to SGBS adipocytes for 24 h did not improve glucose uptake with or without the presence of diverse doses of insulin. Nevertheless, acute high dose administration of GIP without prior use of insulin caused the glucose uptake to rise by 2-fold. Because this experiment was performed in the absence of insulin, it was considered possible that the observed effects of GIP were a consequence of this absence. However, studies on the effect of insulin on GIP-stimulated glycerol release revealed that when cells were incubated in the presence of 10^{-10} M insulin, a concentration equivalent to that found circulating early postprandially, insulin resulted in a concentration-dependent inhibition of the GIP effect on lipolysis. (Flakoll *et al.,* 1996). This suggests that factors modulating glucose metabolism *in vivo* are not present *in vitro* and that *in vivo* factors are necessary for the full mechanism.

6.4.2 Effects of Ghrelin on glucose uptake in SGBS adipocytes

Ott *et al.,* (2002) reported that ghrelin does not stimulate glucose uptake without insulin in brown adipose tissue. The data in the present study, however, showed that, in SGBS adipocytes, insulin-independent (basal) glucose uptake is significantly increased in response to ghrelin and that administration of ghrelin without the presence of insulin augmented glucose uptake in a dose-dependent manner. Nevertheless, after applying different doses of insulin to the treated cells the glucose uptake was significantly down regulated compared with control untreated cells. This might indicate that ghrelin has a 'insulin like' rather than 'insulin-sensitising' effect. Besides, ghrelin did block the development of insulin sensitivity induced by acute exposure to varying concentrations of insulin.

Evidence is rapidly accumulating that indicates a pivotal role for ghrelin, not only in the regulation of growth hormone secretion but also in the control of energy homeostasis and food intake (Nakazato *et al.,* 2001). However, peripheral actions of this stomach hormone particularly on tissues that contribute to the control of energy homeostasis are poorly understood, although a previous Chapter of this thesis showed that the ghrelin receptor is expressed in adipocytes. And other studies have described the stimulatory effect of ghrelin on pancreatic insulin secretion (Date *et al.,* 2002) and insulin signaling in hepatoma cells (Murata *et al.,* 2002).

A possible physiological action for ghrelin may be associated with the previous study that specific circulating fatty acids are essential for optimal glucose stimulation of insulin secretion following fasting (Stein *et al.,* 1996). Ghrelin could be capable of stimulating lipolysis under conditions in which insulin levels are of insufficient magnitude to inhibit its action, and this may ensure that levels of circulating FFAs are optimal for glucose and ghrelin stimulated insulin secretion. Such a proposal is open to experimental verification. Horeover, additional *in vitro* and *in vivo* studies are required to investigate the physiological relevance of the effects of ghrelin on glucose uptake and or insulin signaling pathway in fat cells.

6.4.3 Effects of Obestatin on glucose uptake in SGBS adipocytes

Obestatin stimulation alone did not have any effect on basal glucose uptake, Furthermore, pre-treated SGBS cells with 100 nM obestatin in the presence of different concentration of insulin did not alter the insulin-induced glucose uptake effect compared with the untreated adipocytes.

The data of this experiment do not support a role for obestatin on an adipocyte insulin activated endpoint such as glucose uptake. This finding is in agreement with a previous report (Green *et al.,* 2007) which observed that obestatin peptide has direct and indirect effects on food intake, but no direct actions on glucose homeostasis or insulin secretion in mice.

6.4.4 Effects of insulin and GLP-1or Ghrelin on GLUT4 mRNA expression

Having established the stimulatory effect of both GLP-1 and ghrelin on glucose uptake, whether this was mediated through increased expression of glucose transporter GLUT4 was examined.

This study demonstrated that a minor decrease of GLUT4 mRNA levels was only observed in the low dose GLP-1 treatment, whereas GLUT4 mRNA was not altered at the high GLP-1 dose or with the ghrelin treatment.

The considerable stimulatory effect of GLP-1 on glucose uptake in SGBS adipocytes is supported by a similar observation in murine 3T3-L1 cells (Egan *et al.* 1994). The presence of GLP-1 for 24 h was reported to nearly double the content of both GLUT4 and GLUT1 protein levels in 3T3-L1 adipocytes compared with control cells; however, mRNA levels for both transporters was not altered.

It has been suggested that the prolonged exposure of cultured adipocytes to insulin results in decreased levels of GLUT4 mRNA (Flores-Riveros *et al.*, 1993) and down-regulation of GLUT4 glucose transporters at the plasma membrane (Kozka *et al.* 1991). However, primary cultures of rat adipocytes also have reduced levels of

GLUT4 even when no insulin is added to the medium (Hadjuch *et al.*, 1992; Gerrits *et al.*, 1993), indicating that insulin is not the only factor modulating facilitative glucose transporters.

This is the first demonstration of the stomach-derived hormone ghrelin on modulation of transporter GLUT4 in an insulin-sensitive tissue, SGBS adipocytes.

In summary, the studies presented in this chapter indicate that specific gut hormones, GIP, GLP-1, obestatin and ghrelin in addition to their well known effects on distant target sites to promote the efficient uptake and storage of energy, may have further effects on major targets of insulin action such as white adipose tissue. However, it should be noted, that the effect of several tests revealed subtle differences (P-values $P<0.05$ or $P<0.01$) when multiple comparisons were made. There is a possibility that some of these differences have arisen by chance (Type 1 error). More work including the influence of these gut peptides on glucose uptake by fat cells is needed in the future using larger sample sizes to justify that the effect did not just happen coincidentally.

Nevertheless, this study may assign a novel role for adipose tissue as an important relay station in mediating those gut hormone effects on adipocyte metabolism and on energy homeostasis.

CHAPTER 7
GENERAL DISCUSSION

7.1 Introduction

The main work presented in this thesis was on the expression and action of a range of gut hormone receptors in human white adipose tissue. The results presented in Chapters 3 to 6 have shown that gut hormone receptor genes and proteins including these for the incretins, GIP, GLP-1, and for ghrelin and obestatin are expressed by both mouse and human white adipocytes, and this selection of gut peptides contribute to the regulation of a number of adipokines including classical hormones and cytokine gene expression in human adipocytes.

Additionally, GLP-1 and ghrelin appear to have effects on insulin action in white adipose tissue. The techniques utilized in the whole study included conventional RT-PCR and real-time PCR for analysis of receptor and adipokine gene expression respectively, western blotting for investigation of the receptor protein expression, electrospray ionization mass spectrometry for obestatin peptide molecular weight examination, and 2-deoxy-D-glucose for detection of the glucose uptake in human adipocytes.

7.2 ROLE OF INCRETINS IN WHITE ADIPOSE TISSUE

7.2.1 Incretin receptor gene expression in white adipose tissue

The present study demonstrates that GIPR mRNA was expressed in the major white adipose tissue depots of both mice and humans. On the other hand, differences in GLP-1R gene expression between subcutaneous and visceral fat were shown in both mouse and human tissues. GLP-1R gene expression was found in the omental but not the subcutaneous WAT of the human samples.

The findings presented here, however, could be the result of a different regulatory mechanism of subcutaneous and visceral fat gene expression by multiple stimuli. Adipose tissue located within the abdominal cavity has been suggested to be functionally and metabolically distinct from that of the subcutaneous compartment and these differences could play a role in obesity-related complications (Vohl *et al.*,

2004). Tchkonia *et al.,* (2006), demonstrated regional differences in the intrinsic characteristics of the preadipocytes in the different fat depots, with those of subcutaneous adipose tissue presenting greater differentiation and fat cell gene expression but less apoptosis than that of visceral fat tissue. Moreover, in recent years, it has been shown that the promoter region of the incretin receptor genes contains multiple Sp1/Sp3 binding sites, which appear to be involved in the cellular expression of the receptor (Barth *et al.,* 2002).

The present study also showed the presence of GLP-1R mRNA in a range of non-adipose mouse tissues including skeletal muscle, small intestine, liver, kidney and brain. GLP-1R gene expression was detected not only in mouse liver, but also in human liver tissue, which supports the outcome of two studies (Schmidtler *et al.,* 1994; Villanueva-Peñacarrillo *et al.,* 1995). The known insulin secretion-independent effect of GLP-1 on hepatic glucose uptake is consistent with the presence of specific GLP-1 receptors on liver cells. Binding of GLP-1 to these receptors could initiate intracellular signaling pathways that could target kinases and/or factors involved in glucose uptake and glycogen synthesis.

Both incretin receptor genes are expressed in differentiated mouse and human white adipocytes. Western blot studies confirmed the expression of both GIP and GLP-1 receptor proteins in SGBS adipocyte cultures similar to the mRNA data. The receptor genes are expressed after differentiation but not in preadipocytes. The signals for the GIP and GLP-1 receptor mRNA and protein are late post differentiation.

The presence of these gut peptide receptors in fat cells could be an indicator of physiological effects mediated by these gut peptides in adipose tissue. To date, surprisingly little is known about these gut peptides interactions with adipocyte biology. However, GLP-1 seems to exert differential, concentration-dependent effects on lipid metabolism in human adipocytes, which may be explained by the existence of different GLP-1-binding receptors (Villanueva-Penacarrillo *et al.*, 2001).

7.2.2 The regulation of adipokine production by the incretins

Incretins may modulate the production of adipocyte-derived adipokines; adiponectin mRNA level increased after applying high doses of either GLP-1 or GIP for 24 h in differentiated SGBS cells. Adiponectin is one of the most abundantly expressed adipose-specific proteins (Maeda *et al.,* 1997), and is implicated in the pathogenesis of obesity and insulin resistance in rodents and humans (Kim *et al.,* 2002).

In addition to the most remarkable ability of incretins to excite insulin secretion in the presence of glucose stimulatory concentrations (Kreymann *et al.,* 1987), the role of incretins in regulating glucose and lipid metabolism has been reported by others (Hauner *et al.,* 1988; Wang *et al.*, 1997). The present data reveal that both GLP-1 and GIP appear to have a small stimulatory effect on adiponectin mRNA levels at higher concentrations in human fat cells. It should be noted, however, that the effect of incretins on adiponectin is very small, and further work is required on the influence of both incretins on adiponectin release by fat cells. Furthermore, it is important to determine whether the functional hormone is secreted from the adipocytes.

The stimulation of MCP-1 by GIP indicates that GIP may be involved in the synthesis of this adipokine in adipocytes. This stimulatory effect contradicts the proposed anti-obesity function of GIP (Gault, *et al.,* 2003). Moreover, observations from a recent study in transgenic GIP receptor-deficient mice indicated that GIP directly links overnutrition to obesity, therein playing a vital role in the development of obesity and related metabolic disorders (Miyawaki *et al.,* 2002). This study with differentiated SGBS adipocytes has contributed significantly towards research in this area.

The present results with 24 h of GIP treatment in human adipocytes, showed that the expression of IL-6, another pro-inflammatory cytokine, was stimulated in a similar pattern. This indicates that GIP peptide is a regulatory agent that may have a role in the inflammatory response in white adipose tissue.

GLP-1 treatment did not alter the level of MCP-1 gene expression in human cell culture systems. However, a very small but statistically significant increase in IL-6 mRNA level was only observed with high doses of GLP-1 treatment, whereas IL-6

mRNA remained unchanged at lower doses.

The data in this thesis may suggest that in general, regulation by incretins in SGBS adipocytes is multi-factorial, and most likely the result of a complex interplay of different hormones and cytokines.

7.2.3 The effect of Incretins on insulin sensitivity in SGBS adipocytes.

GLP-1 enhances the metabolic responses to insulin in white adipose tissue. This study with differentiated SGBS adipocytes showed that GLP-1 treatment had a clear dose-dependent increase in glucose uptake with or without acute insulin-stimulation when compared with control cells. This significant stimulatory effect of GLP-1 on glucose uptake in SGBS adipocytes is supported by a similar observation in murine 3T3-L1 cells (Egan *et al.* 1994), indicating that the actions of insulin to stimulate glucose uptake and its utilization for lipid synthesis were enhanced by the presence of GLP-1. Furthermore, a very recent *in vivo* study in humans has also shown that GLP-1 has effects on glucose disposal (Meier *et al.,* 2006).

The study also demonstrated a minor decrease of GLUT4 mRNA levels, which was only observed with low dose GLP-1 treatment. The presence of GLP-1 for 24 h was reported to almost double the content of both GLUT4 and GLUT1 protein levels in 3T3-L1 adipocytes compared with control cells (Egan *et al.* 1994). However, mRNA levels for both transporters were not altered (Egan *et al.* 1994). Moreover, primary cultures of rat adipocytes also have reduced levels of GLUT4 even when no insulin was added to the medium (Hadjuch *et al.*, 1992; Gerrits *et al.* 1993), indicating that insulin is not the only factor modulating facilitative glucose transporters.

Although the insulin-releasing action of GIP makes a major contribution to its metabolic role, in this study however the addition of 100 nM GIP to SGBS adipocytes for 24 h did not improve 2-deoxy-D-glucose uptake with or without the presence of different doses of insulin. Nevertheless, the acute administration of high dose GIP without prior use of insulin caused an increase in glucose uptake.

Studies on the effect of insulin on GIP-stimulated glycerol release revealed that when cells were incubated in the presence of insulin, a concentration equivalent to that found circulating early postprandially, insulin resulted in an inhibition of the GIP effect on lipolysis. (Flakoll *et al.,*1996). This study suggests that factors modulating glucose metabolism *in vivo* are not present *in vitro* and that *in vivo* factors are necessary for the full response.

In conclusion, both incretin hormones, GIP and GLP-1, appear to interact with adipocyte functions. Regulation of endocrine adipocyte function and enhancement of insulin action may be an important direct effect.

7.3 ROLE OF GHRELIN IN WHITE ADIPOSE TISSUE

7.3.1 Ghrelin receptor gene expression in white adipose tissue

Ghrelin receptor, GHS-R, gene expression was detected in the mouse brain and in major white adipose tissue depots of both mice and humans, which is consistent with the observations of previous studies (Korbonits *et al.*, 2004; Kojima *et al.*, 1999; Kim *et al.*, 2004).

By using 3T3-L1 cells in culture, the current study indicated that the GHS-R gene is expressed after differentiation, although not in preadipocytes and these findings were further supported by exploring GHS-R gene and protein expression in human SGBS adipocyte cultures. The signals for the GHS-R mRNA were late post differentiation; emerging from day 8 and onwards.

To date, surprisingly little is known about the interaction of ghrelin with adipocyte biology. However, recent studies have shown that ghrelin activates the mitogen-activated protein kinase pathway *in vitro,* which stimulates cellular proliferation and differentiation in cultured white adipocytes (Kim *et al.*, 2004; Zhang *et al.*, 2004). In contrast, using brown adipocytes, ghrelin does not seem to affect adipocyte differentiation (Ott *et al.*, 2002).

7.3.2 The regulation of adipokine production by ghrelin

Little is known concerning the regulation of adipokine production by ghrelin. However, a recent study has examined the possibility that ghrelin directly suppresses adiponectin mRNA expression in brown adipocytes (Ott *et al.*, 2002). The data in this thesis does not support such a role in the case of white fat cells.

Interestingly, however, ghrelin treatment for 24 h resulted in a statistically significant inhibitory effect on MCP-1 mRNA levels by 50% in both intermediate and high doses. The results in this thesis were further strengthened by an independent study, in which ghrelin was found to have potent anti-inflammatory effects in human endothelial cells, which are likely to be mediated by inhibition of NF-κB activation (Li *et al.,* 2004). These findings raise the possibility that reduction in endogenous ghrelin could potentially contribute to the pro-inflammatory state and the increased incidence of atherosclerosis in obese patients.

The present data demonstrate an inhibitory effect of ghrelin on the expression of pro-inflammatory cytokine, MCP-1 in human adipocytes supporting a possible regulatory role for ghrelin in a range of inflammatory conditions associated with obesity.

7.3.3 The effect of ghrelin on insulin sensitivity in SGBS adipocytes.

The data in this thesis support a direct role for ghrelin in enhancing insulin-independent glucose uptake in SGBS adipocytes. A significant increase in 2-deoxy-D-glucose uptake occurred at high ghrelin concentration; nevertheless, after applying different doses of insulin the glucose uptake was significantly down-regulated compared with control untreated cells.

The present study also showed that acute administration of ghrelin without the presence of insulin augmented glucose uptake in SGBS adipocytes in a dose-dependent manner and was significant at the higher doses. This suggests that ghrelin has 'insulin-like' rather than 'insulin-sensitising' effects. Ott *et al.,* (2002) reported that ghrelin did not stimulate glucose uptake without insulin. However; the study in this thesis suggested the opposite.

A possible physiological action of ghrelin might be the stimulation of lipolysis under conditions in which insulin levels are inadequate magnitude to inhibit its action, and this may ensure that levels of circulating FFAs are optimal for glucose and ghrelin stimulated insulin secretion. Such a proposal is open to experimental verification.

Moreover, the current study showed no change in GLUT4 mRNA levels, with ghrelin treatment. It was not possible to identify the precise mechanism by which ghrelin stimulates glucose uptake. Follow-up work would need to investigate the signal transduction pathway using cultured cells.

7.4 ROLE OF OBESTATIN IN WHITE ADIPOSE TISSUE

7.4.1 Obestatin receptor gene expression in white adipose tissue

The gene expression of the putative receptor for the anorexigenic peptide obestatin, GPR39, occurred in a range of mouse tissue including skeletal muscle, small intestine, liver, kidney and brain, as well as white adipose tissue of both mice and humans. The tissue expression pattern of GPR39 indicates that this receptor might be truly essential for the function of a number of metabolic organs.

The current study further indicates that the GPR39 gene is indeed expressed after differentiation but not in preadipocytes in cell culture systems (mouse 3T3-L1 and human SGBS cells). The signal was immediately observed at day 1 post-induction and was present throughout the subsequent time course shortly after differentiation. This finding is consistent with GPR39 protein expression in human SGBS adipocytes.

The G-protein coupled receptor, GPR39, has been proposed to be the target receptor of obestatin (Zhang *et al.,* 2005). However, this has not been confirmed by other studies (Holst *et al.,* 2007; Lauwers *et al.,* 2006). This is the first occasion in which both mouse and human adipocytes cell culture systems were found to express the transcript coding for the proposed obestatin receptor (GPR39). Moreover, these pre-adipocyte cell culture systems provide a valuable model for analysing ligand signalling by obestatin, for testing different obestatin agonists and antagonists and for the physiological nature of the effects mediated by obestatin in adipose tissue.

Although, Tnag *et al.* (2008) demonstrated that obestatin induced cell proliferation in a dose-dependent manner with MEK/ERK 1/2 phosphorylation in primary cultures of piglet adipose cells, another study showed that obestatin did not stimulate cell cycle or viability of the murine adipocytes cell line 3T3-L1 and inhibited the proliferation and differentiation of 3T3-L1 preadipocytes (Zhang *et al.,* 2007). Furthermore, the effect of obestatin was contrary to that of ghrelin on these adipocyte cell line (Zhang *et al.,* 2007).

7.4.2 The regulation of adipokine production by the obestatin

Surprisingly only two previous studies have evaluated the effect of obestatin in adipocyte biology. No information has been reported on the relationship between obestatin and adipokine production. However, the present results with 24 h treatment with obestatin from different sources in human adipocytes, including des-obestatin (11–23), showed a stimulatory effect in pro-inflammatory adipokine (MCP-1 and IL-6) mRNA levels. This indicates that obestatin and des-obestatin peptides may act as regulatory agents that have a role in the inflammatory response in white adipose tissue. However, more work, including investigations on the influence of obestatin and des-obestatin on inflammation-related adipokine release by fat cells is needed in the future.

Des-obestatin (11–23) showed a marked stimulation of mRNA levels for leptin and adiponectin. A very recent *in vivo* study reported that plasma obestatin concentrations are negatively correlated with body mass index, insulin resistance index, and plasma leptin concentrations in obese humans (Nakahara *et al.,* 2007). Of course, des-obestatin is a small fragment of obestatin, and may be part of a system with multiple effector elements, which not only have opposite actions, but also regulate the action of each other.

7.4.3 The effect of obestatin on insulin sensitivity in SGBS adipocytes.

Obestatin stimulation alone did not have any effect on basal glucose uptake in human SGBS adipocytes. Furthermore, pre-treated SGBS cells with obestatin in the presence of different concentrations of insulin did not alter the insulin-induced glucose uptake effect compared with the untreated adipocytes.

The current data do not support a role for obestatin in adipocyte insulin activated endpoints such as glucose uptake. This finding is in agreement with a previous report (Green *et al.,* 2007), which observed that obestatin peptide has direct and indirect effects on food intake, but no direct actions on glucose homeostasis or insulin secretion in mice.

7.5 ROLE OF GASTROINTESTINAL HORMONES IN OBESITY

The expression of the GIPR, GLP-1R, GHS-R and GPR39 genes was evident in four obese human (subcutaneous) WAT.

The presence of GLP-1R expression in subcutaneous fat of obese subjects suggests that an exaggerated 'incretin' factor may have a pathophysiological role in obesity. Moreover, the hyper-secretion of GLP-1 may be the long sought 'incretin' factor in obesity. Studies suggest that obesity often produces a type 2-like diabetic state, which resolves after weight loss (Molavi *et al.,* 2006).

Importantly, the putative obestatin receptor, GPR39, gene expression was found in the subcutaneous fat of all four human obese subjects, but no expression was evident in lean subjects. It could be hypothesised that high expression of the GPR39 gene in obese subjects is due to a decreased obestatin level in obesity. In a recent study, Huda *et al.*, (2008) have shown that plasma obestatin concentrations are significantly lower in obese subjects when compared to lean controls. Nevertheless, the reduction of this peptide in obese humans is probably maladaptive and may further propagate the obese state.

Obesity has emerged as a low-grade inflammatory state and it is still unclear whether adipocytes or the SV fraction in WAT have a more prominent role in the secretion of the major pro-inflammatory factors (Fain *et al.,* 2004). A remodelling of WAT by cross-talk between these two fractions is likely to mediate the low-grade inflammatory response in obesity.

7.6 CONCLUSION AND PERSPECTIVES

The studies described in this thesis have established that the genes encoding the receptors for ghrelin (GHS-R), obestatin (GPR-39), glucagon-like peptide-1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIPR) are expressed in major white adipose tissue depots and are indeed expressed in adipocytes. The foundation has been laid for future work, which, in first instant, could involve further and more detailed examination of the metabolic role of these gut hormones in WAT using a multiplicity of approaches.

Furthermore, the relationship between obesity and gut hormones remains to be clarified; additional studies could be aimed both *in vivo* and *in vitro*. It is very important to elucidate the relationship between the serum levels of these gut hormones and adiposity in both obese and lean subjects.

The data in this thesis also suggest that GLP-1, in addition to its well-known effects on insulin secretion by pancreatic β cells and glucagons secretion by α cells, may have effects on major targets of insulin action sites such as white adipose tissue. The presence of mRNA for the GLP-1 receptor in liver, skeletal muscle as well as adipose tissue raises the possibility that binding of GLP-1 to these receptors could initiate intracellular signaling pathways that could target kinases and/or factors involved in glucose uptake and glycogen synthesis.

The current study has provided evidence for selective effects of ghrelin on adipocyte function and thus proposes a role for adipose tissue as a novel mediator of ghrelin's effects on energy balance and glucose homeostasis. Moreover, the fact that this peptide can inhibit the production of pro-inflammatory "MCP-1" in human fat cells

suggests a potential mechanism through which ghrelin may protect against pathologic inflammatory conditions in obesity. However, it should be noted, that appetite is regulated by numerous factors that may interact with and compensate for each other. Therefore the effectiveness of such an approach has to be directly shown in clinical studies. Further research is required to investigate whether ghrelin antagonists or agonists might be viable anti-obesity or malnutrition-treating drugs that can be used in wide clinical practice.

It is now recognised that adipose tissue is an active endocrine organ, secreting a wide range of adipokines and other factors, which can act locally within the tissue and may also be released into the general circulation, with effects on multiple organs and systems. The work presented in this thesis provided some evidence that incretins, obestatin and ghrelin regulate the expression and release of key adipokines and thereby play a significant role in adipose tissue as part of the cross-talk between the gut and adipose tissue.

APPENDIX

Part of the work in this thesis has been presented in the following publications and oral communications:

Publications

1. Al-naimi S, Ranganath LR, Shenkin A & Trayhurn P (2006). Do white adipose adipocytes express receptors for gut hormones? (Abstract). *Ann Clin Biochem (Supplement)* **43,** S14

2. Al-naimi S, Ranganath LR & Trayhurn P (2007). Ghrelin and Obestatin and the white adipose tissue. (Abstract). *Ann Clin Biochem (Supplement)* **44,** S77

Oral presentation

1. "Do white adipose adipocytes express receptors for gut hormones?" (2006) Focus, The Association of clinical Biochemistry, Annual national meeting, Brighton, UK.

2. "White Adipocytes Express Receptors for the Gut Hormones Ghrelin, GIP, GLP-1 and Obestatin" (2006) International Congress on Obesity, Sydney, Australia.

3. "Ghrelin and Obestatin and the white adipose tissue"(2007) Focus, The Association of clinical Biochemistry, Annual national meeting, Manchester,UK.

4. "Gut Hormones ,Ghrelin and Obestatin in the white adipose tissue"(2008) The Association of clinical Biochemistry, NW ACB Ian Ward Meeting, Birchwood centre, Warrington, UK.

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